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Infection and immunity in the Pacific white shrimp, *Litopenaeus vannamei*

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

Aquaculture has become one of the most rapidly growing sources of food animal protein in the world. Decreasing stocks within wild fisheries and increasing demand for seafood are driving this precipitous growth mostly from the United States, Europe, and Japan. More recently demand in developing economies has grown at a rapid pace. One of the fastest growing market segments within aquaculture is farm-raised shrimp. According to data from the United Nations Food and Agriculture Organization (FAO), the last twenty years has seen farmed shrimp production of *Litopenaeus vannamei,* commonly known as the Pacific white shrimp or the whiteleg shrimp, rise from 8000 metric tons produced in 1980 to 1,380,000 metric tons produced in 2004.[13] Most of this rapid increase in production can be accounted for by growth in culture throughout Southeast Asia, as these countries began to intensively culture imported *L. vannamei* in lieu of the native *Penaeus monodon,* the black tiger shrimp*,* for export. Countries with rapid growth of *L. vannamei* production include countries such as China, Thailand, Indonesia, and Vietnam.[13] Domesticated stocks of *L. vannamei*, a species native to the west coast of South and Central America, were perfectly suited to culture conditions in Asia. *L. vannamei* proved to be faster growing, more adaptable to pond conditions, had less stringent dietary requirements, and most importantly, was amenable to much higher stocking densities. The culture of farm-raised shrimp for export to the US and Europe provide a very important source of foreign currencies for many of these developing countries and thus, expansion of the industry was pursued quite rapidly.[56]

Following the introduction of exotic *L. vannamei* into Asia as the first domesticated shrimp species, it rapidly became the dominant species worldwide, where in 2004 it accounted for half of the farm raised shrimp produced globally.[24] Subsequently, demand in the United States and the European Union rose as a more affordable shrimp came onto the market. For example, shrimp imports rose from $1.6 billion to $3.7 billion from 1990-2004[52] representing 34% of total seafood imports and 25% of total seafood consumption in 2004. As of 2004, 70% of the United States seafood was imported with 40% of it being farm-raised in origin.[52] Rapidly expanding production of *L. vannamei* outstripped demand and led to price depression in international markets, mostly in the United States and European Union. Farm gate value for 15–20 gram size *L. vannamei* has steadily decreased from $5 in 2000 to about $3 in 2005.[13] Per capita consumption of seafood products by consumers has also seen a strong increase over this time period. In 2006, the average per capita consumption of seafood in the United States was 16.5 pounds, with 4.4 pounds of this consisting of shrimp.[4] Current consumer demand for seafood has remained high with per capita consumption of shrimp being 4.1 pounds per year in 2009, all while per capita consumption of all other levels of animal protein in the United States have been in decline, with total consumption dropping in a six year period from 237 pounds in 2004 to 224 pounds in 2010.[33]

As farm-raised shrimp production of *L. vannamei* has increased in market share and industry size, the impact of disease on production and profitability has also increased. Producers have adopted practices such as higher stocking densities, smaller inland lined pond culture, and higher feeding rates using genetically selected animals for higher growth to increase competitiveness and increase production. This increase in intensity,

and thus higher stocking densitities has led to an increasing vulnerability to infectious diseases, specifically viral pathogens and bacterial infections.[55] In addition, such a new and rapidly growing industry in developing economies often does not have the regulatory apparatuses in place to prevent importation of new infectious diseases with stocks of live animals. For example, pathogen translocation and disease outbreaks have ensued with significant economic losses immediately following the emergence of each of the major viral pathogens[55] often crossing global hemispheres in a very short period of time.

Infectious diseases caused by viral pathogens can cause substantial mortality in *L. vannamei* and result in devastating financial losses. It has been estimated that annual global losses, mostly viral in etiology, have been as high as $3 billion a year.[27,56] The impact of disease as an impediment to growth in this new production system and species cannot be understated and thus, novel research that may mitigate these disease losses or assist in disease prevention is crucial.

The objectives of the research described within this dissertation are focused on developing disease models and model systems for viral and bacterial pathogens in the commercially important, farm-raised marine shrimp species, *L. vannamei.* Following the establishment of these models is the development and evaluation of RNA interference (RNAi) based technology for use as both a prophylactic and therapeutic intervention against disease. In other words, could a scientific approach be used to model relevant infections with different pathogenic organisms in this species? Can these models then be used as a foundation to develop antiviral prophylaxis or therapeutics based upon a newly described RNAi based specific immune response?

The first paper describes the development of a propagation system for use in conjunction with a challenge model for a bacterial pathogen, the Necrotizing Hepatopancreatitis Bacterium (NHPB). This paper provides the foundations for conducting experimental challenge studies using an obligate intracellular bacterium that cannot be cultured *in vitro* and therefore must be propagated *in vivo*.

The second paper describes the development of a viral disease challenge model using a newly emerged viral pathogen, Infectious Myonecrosis Virus (IMNV), as well as the use of RNAi based antiviral molecules to prevent mortality caused by this disease. It also describes a novel method to optimize an RNAi antiviral molecule by down-selection of sequences in order to elucidate the minimum requirement for a successful RNAi antiviral. Additionally, this manuscript evaluates the dosage requirements and duration of the protective response observed using a down-selected RNAi antiviral molecule.

Finally, the third paper examines the application of these aforementioned down-selected RNAi antiviral molecules as a therapeutic treatment against preexisting IMNV infection. The objective of this work was to ascertain if these triggers could not only prevent disease, but also be used as a therapeutic treatment which may have application in the field during an acute outbreak or latent infection within a pond system.

Dissertation Organization

This dissertation is organized in a journal paper format. Chapter 1 includes a brief introduction to the topic followed by a review of the literature, with a focus on current knowledge of the modeled disease organisms and disease intervention strategies being evaluated and explored in shrimp infectious disease research. Chapters 2, 3, and 4 are the

author's research projects prepared in manuscript format for publication. John Dustin Loy was the primary researcher and author of all manuscripts with assistance from the co-authors as listed. Chapter 5 contains the general conclusions and implications of the conducted research followed by an acknowledgments section.

Literature Review

White Spot Syndrome Virus: The first shrimp disease pandemic

White spot syndrome virus (WSSV) was one of the first economically significant diseases in shrimp production to emerge and become pandemic, resulting in worldwide losses in the billions of dollars. WSSV was first observed in 1992 after several outbreaks of a high mortality disease of viral origin in cultured *Penaeus japonicus* occurred in northern Taiwan. In 1993 the disease was seen in the black tiger prawn, *P. monodon*.[6] This disease quickly spread across the Asian continent having a tremendous impact on the shrimp industry. It is estimated that Asia alone has lost over $6 billion in production since 1992. Following the introduction of WSSV into the Americas (Ecuador) in 1999, it accounted for losses of $1-2 billion in a few years time.[27] This disease translocation was devastating to the shrimp industries in countries with high levels of shrimp culture. For example, Ecuador alone experienced dramatic losses with a 65% loss in production observed immediately after the introduction of WSSV into the country. This accounts for, in lost exports alone, over $500 million. In addition, 130,000 jobs were lost and over 100,000 hectares of ponds were left abandoned as WSSV made it impossible to profitably culture shrimp.[32] Similarly, Peru experienced a precipitous drop in production to one tenth of the production levels seen in 1998, just two years following the introduction of

WSSV. In Peru in 2000, 85% of shrimp ponds had been abandoned as producers had accumulated $9 million in lost feed costs.[32] In China, it was estimated that 80% of total production losses annually were and continue to be attributed to WSSV outbreaks.[64] The vast majority of these losses occur because a pathogenic virus in a pond of growing shrimp forces the grower to flush out a stocked pond, prematurely harvest (if the animals are marketable), and increase production costs through decreased feed conversion. Moreover, this can be a complete loss of animals from mortality if the disease is not diagnosed rapidly.[55]

Necrotizing Hepatopancreatitis: and emerging bacterial pathogen

In addition to viral diseases, diseases with bacterial etiology remain a serious concern for shrimp farmers. However, often these disease problems exist as secondary infections in healthy animals or are food safety issues, such as observed infections or colonization with *Vibrio sp*. Few primary bacterial pathogens have been characterized or described in shrimp. However, at least one primary pathogen has been found to be associated with disease in cultured *L. vannamei*, called necrotizing hepatopancreatitis bacterium (NHPB). The syndrome caused by NHPB, necrotizing hepatopancreatitis (NHP) was first reported on a shrimp farm in Texas in 1985 and has since been demonstrated in cultured shrimp in Peru, Ecuador, Venezuela, Brazil, Panama, Costa Rica, and Mexico where it has caused significant mortalities in affected ponds.[9,25,28]

NHPB is a gram-negative, pleomorphic, obligate intracellular bacterium.[15] Phylogenetic analysis of 16S rRNA has placed it in the α-subclass of Proteobacteria, along with such pathogens as Rickettsia (~83.5% nucleotide identity).[29] Clinical signs of infection include a reduction in feed intake and empty guts, softened shells, flaccid

muscle tissue, epicommensal fouling, darkened gills and pleopods, and atrophy of the hepatopancreas organ.[25] The hepatopancreas organ is the primary site of infection for NHPB, and as such it undergoes changes over the course of infection. In severe NHPB infection, the hepatopancreas may develop gross black streaks indicating the melanization of hepatopancreatic tubules. However, few, if any, of these gross lesions are pathopneumonic for NHP, making confirmatory testing by more specific molecular or immunologic tests such as Polymerase Chain Reaction (PCR) or immunohistochemistry prudent. Histopathological characteristics of NHPB infection include hepatopancreatic atrophy and multifocal granulomatous lesions. Lipids in resorptive (R) cells are reduced and the number of blister-like (B) cells is drastically reduced or absent in the hepatopancreatic tubules. Cells present in the granulomatous lesion may be hypertrophied and contain masses of pale, basophilic non-membrane bound NHP-bacteria free in the cytoplasm. Secondary *Vibrio* infections, have been observed in some severe NHPB infections, complicating disease diagnosis and obscuring the primary cause of disease.[25]

NHPB causes a disease in shrimp characterized by a 2-6 week incubation period followed by high mortality that peaks at approximately 34 days post exposure.[54] In experimental infections using individual exposures of *L. vannamei*, Vincent and Lotz[54] observed stage I at 6 to 23 d post-exposure, stage II at 16 to 37 d post-exposure, and stage III at 16 to 51 d post-exposure. The timing of stage III of NHP disease corresponded to observed mortalities from infection.[54] Further work examining the time course of NHP disease, modeling of pond conditions favoring the disease, and development of an *in vivo* propagation method is further discussed in Chapter 2 of this dissertation.

Although this agent is currently restricted to the Americas, the opportunity for worldwide spread of NHPB is high. Frozen whole shrimp may provide a means for spread, as it is possible to propagate infections via frozen tissue[7]. In addition, concerns over antibiotic residues have resulted in disuse of antibiotics in many areas, the only available intervention for NHPB. Currently, little is understood about the life cycle or etiology of NHPB outside the shrimp host and more research into this area could help guide a prevention and control program in infected regions.

Disease Prevention and Control in farm systems

The very high risk, or potential risk, for tremendous losses associated with a disease outbreak has led to the development of various control strategies. The most important of these has been the utilization of a concept called "stock control." Stock control in shrimp production has been defined by Dr. Donald Lightner as "the use of captive or domesticated stocks, cultured under controlled conditions, and which have been the subject of an active disease surveillance and control program."[24] Although this practice is quite common among commercial operations in a variety of other farm-raised species, stock control was not initially pursued aggressively in shrimp culture. This may be because traditional shrimp culture methods, practiced in many countries, relied on wild seed or post larvae to stock the ponds, and farmers felt little need to adopt these practices without cause.

Once a controlled culture system with disease free stocks is in place, other disease control practices focus largely on pathogen exclusion by stocking specific pathogen free (SPF) larvae or postlarvae, decontamination and filtration of water to prevent pathogen, pathogen vectors, or wild shrimp introduction, and strict biosecurity at

the hatchery and pond sites.[24] These control strategies can be effective as long as virus remains excluded from the culture system. However, this has become challenging due to the prevalence of WSSV and other significant pathogens that have become endemic in wild or extensively managed shrimp species in the estuarine waters surrounding shrimp farms as well as the heavy use of live feeds in broodstock maturation diets.

Viral outbreaks also cause devastating financial losses due to such acute mortality in these naïve susceptible SPF populations. Complicating matters, viruses can also manifest as a low-level persistent infection without clinical signs in healthy animals, but stress or lower temperatures can trigger disease and mass mortalities.[56] Without robust and vigilant diagnostics the disease can become widespread on pond sites and farms resulting in the potential for massive losses of animals in a short period of time. Currently, there are no truly effective interventions or therapeutic treatments for viral disease in shrimp farms, though some have shown promise experimentally[10] and are described in detail later in this review.

Following on the WSSV pandemic of the early 1990s in Asia and late 1990s in the Americas, the theme of a newly emerging disease causing massive untreatable losses and being spread across the globe continues to repeat itself with several viral pathogens, including most recently, infectious myonecrosis virus (IMNV).

Infectious Myonecrosis Virus

In 2002, an outbreak of a novel, severe, and unknown disease causing significant mortality and "white muscle" was reported in the northeastern parts of Brazil. This disease was eventually named infectious myonecrosis (IMN) and it caused millions of dollars in losses in Brazil in 2003.[26] Through some unknown mechanism, IMN spread

across the globe to Southeast Asia, to the island of Java, resulting in significant financial losses within Indonesia in 2006.[44] Tremendous losses continue in both of these countries at very high levels, as interventions that worked to exclude WSSV, a large enveloped DNA virus, and the vectors of WSSV, appear to be ineffective at preventing and excluding IMN from shrimp farms.

IMNV disease and pathology

The causative agent for IMN was eventually isolated and named infectious myonecrosis virus (IMNV). IMNV is a non-enveloped, small (40 nm), icosahedral, non-segmented, dsRNA virus, and is a member of the family *Totiviridae.*[37] IMN disease was subsequently reproduced in SPF animals by injection of sucrose density gradient purified IMNV virions, thus fulfilling Rivers' postulates.[38] Mortality attributed to IMNV can range from 40% to 70% over a growout period, with large losses in production that can continue even following a reduction in stocking density. Feed conversion ratio (FCR) can vary from a normal 1.5 to upwards of 4.4[1], causing increases in feed input costs. IMN disease itself is characterized by skeletal muscle necrosis, often grossly described as "white muscle," in the distal abdominal segments followed by mortality, especially following periods of acute stress such as during cast netting or harvesting. Histopathologically, animals demonstrate a characteristic coagulative necrosis of skeletal muscle with fluid accumulation in between muscle fibers, along with pronounced hypertrophy of the lymphoid organ due to accumulation of spheroids.[37] Specific diagnostic tests for IMNV have been developed that include the use of *in situ* hybridization on histopathology slides[37] and a quantitative Real-Time RT-PCR from tissues.[1] Outbreaks in Brazilian farms have been observed in association with the dry

season. An epidemiological survey conducted in Brazil among four farms in an endemic area over the course of a year, found long rearing periods and high stocking densities as two factors highly associated with significant increases in IMN occurrence.[2]

IMNV genome and related viruses

Several *Totiviruses* have recently been discovered in other species including, *Armigeres subalbatus*, a mosquito vector for the parasite *Wuchereria bancrofti*. This virus shares some sequence homology with IMNV with a 29% amino acid identity within the capsid protein and a 44% amino acid identity with the viral RNA dependent RNA polymerase.[63] In addition, deep sequencing of *Drosophila* cell lines revealed 5 additional previously unknown viruses, including a Totivirus with sequence similarity to IMNV, named *Drosophila* totivirus (DTV).[60] Recently, *Leishmania* RNA virus 1 (LRV1), a totivirus of the Trypanosomatid protozoan parasite *Leishmania,* has also been shown to be associated with an increase in the pathogenicity and severity of *Leishmania* parasitic infections *in vivo*[18], indicating that a Totivirus infection within a parasite may modulate host pathogenesis in some manner.

IMNV and other members of the family *Totiviridae* contain two open reading frames (ORFs) in a single genome segment. For IMNV, ORF1 (nucleotides 136-4953) encodes a 1606 amino acid major capsid protein (MCP) and ORF2 (nucleotides 5241-7451) encodes a 736 amino acid RNA dependent RNA polymerase (RdRp)[37]. The IMNV ORF1 sequence encodes a 1605 amino acid polypeptide including the N-terminal sequence of the major capsid protein. The IMNV capsid is isometric with a diameter of approximately 400 angstroms. In addition to these two ORFs, recent studies of the IMNV genome have discovered a “2A-like” cleavage and “shifty heptamer” that may

contribute to formation of a capsid protein-RdRp fusion protein and three putative cleavage protein products of ORF 1.[35] These putative protein cleavage products have been described as Protein 1, Protein 2, and Protein 3 by Nibert (2007). There remains some speculation as to the precise role of these proteins. Protein 1 is a 60 amino acid protein at the N-terminal region of ORF 1, and shares sequence similarities with known dsRNA binding proteins, thus it may be involved in host immune suppression.[48] This is a host evasion strategy seen in RNA viruses in other arthropods. For example, Drosophila C Virus (DCV) and Flock House Virus (FHV) are pathogenic single stranded RNA viruses of other invertebrate species that encode proteins that have dsRNA binding activity with the capacity to modify or inhibit the host RNAi machinery [23,53] and thus suppress the antiviral immune response of the host. Protein 1 may be fulfilling a similar immunomodulatory role for IMNV as to the dsRNA binding proteins characterized in these other invertebrate viruses.

Protein 2 a 32 kDa protein spanning bases 415-1266 and Protein 3 a 38 kDa protein spanning 1267-2247, together representing the first 704 amino acids of ORF1, have been speculated to be candidate minor proteins visualized on viral protein denaturing gels, however this remains speculative in nature.[48] Protrusions of fiber-like densities on the fivefold axis of symmetry were observed in transmission electron microscopic images and further investigated by cryoTEM using 3D reconstruction.[48] These protrusion proteins may be involved in the pathogenesis and transmission of IMNV, which is a fairly uncharacteristic member of the *Totiviridae,* as many members of this family are associated with latent or avirulent infections in the host. Close inspection of these fiber complexes estimated them to be approximately 90 kDa, leading some

authors to speculate that a Protein 2 and Protein 3 heterotrimer may be likely candidates for forming these 5f fiber complexes, but the exact protein structure of these fibers and their role in pathogenesis remain elusive.[48]

Analysis of all the sequence data available identifies a replication strategy similar to one of closest relatives to IMNV, *Giardia lamblia* virus (GLV). In addition to a similar replication strategy, these viruses appear to possess cleavage elements similar to invertebrate infecting members of the segmented dsRNA *Reoviridae.* Experiments conducted using *Giardia lamblia* virus, demonstrated that a specific cell receptor is utilized for virus entry into cells, and that protection was conferred to mutants lacking this receptor.[45] These experiments suggest that a specific cell membrane receptor likely exists within shrimp cells, and a ligand present on the 5- fold fiber complexes of IMNV could provide an avenue for entry into shrimp these cells. Recent work using yeast two-hybrid screens identified shrimp laminin receptor (LamR) as interacting with capsid proteins of IMNV, as well as other shrimp RNA viruses, providing some evidence that this may be a putative cellular receptor for IMNV.[5] Using this data, which underscores the importance of these regions and cleavage products of ORF1, a series of sequence candidates from this region were chosen as RNAi targets in studies described within this dissertation.

Shrimp Vaccination

With the severe impact viral disease has on shrimp farming, there has been a keen interest in developing antiviral prophylaxis or therapies to mitigate disease. Strategies for developing these for shrimp viral diseases have taken many forms. The first has exploited viral envelope proteins in order to interfere with virus/host cell and cell

receptor interactions. This mechanism was hypothesized to be responsible for protection from WSSV observed following the oral administration of formalin inactivated virus.[34] This has since been followed by several other strategies using recombinant protein administration, mammalian derived antisera, DNA vaccination, or delivery via bacterial expression systems.

Protein subunit vaccines to WSSV envelope proteins have been shown to confer protection against WSSV infection in several species of marine shrimp and freshwater crayfish. WSSV contains at least 4 major envelope proteins with no known homology to other virus proteins; these are VP28, VP26, VP24, and VP19. VP28 is present on the outer membrane and is likely involved in cellular entry.[32] VP28 antisera raised in rabbits has been shown to neutralize virus *in vivo*.[32] Recent studies have demonstrated that these four major envelope proteins bind to form a complex, via several pairwise protein interactions and one self-association (via VP28).[66] This provides some evidence that these proteins interact and are likely involved in viral entry into target cells, making them ideal targets as subunit vaccine candidates or for antiviral prophylaxis.

Subunit protein vaccines consisting of both VP28 and VP19 recombinant proteins conferred protection to WSSV infection and protection was seen up to 25 days post administration.[59] More recent approaches have tested co-inoculating shrimp with recombinant cellular receptor proteins and virus, such as using Laminin receptor (Lamr), the protein proposed to be the cellular receptor for Yellowhead virus (YHV) and Taura syndrome virus (TSV), two other highly pathogenic RNA viruses in shrimp.[5] Experiments demonstrated that recombinant laminin receptor (rLamr) produced in yeast protected shrimp from laboratory challenge with YHV when virus and recombinant

protein were co-inoculated.[5] This observation is thought to due to a "blocking" effect mediated by interference caused by rLamr between viral attachment and cellular entry.

DNA vaccines encoding various WSSV envelope proteins have also demonstrated some efficacy in preventing or reducing infection. Naked DNA vaccines for VP28 and VP281 were injected into *Penaeus monodon*, and demonstrable protection was observed for up to 7 weeks following injection.[42] However, injection of naked DNA or plasmid DNA into individual animals is not ideal in a commercial setting due to cost of DNA production and feasibility of individual animal injections in the field. Additionally, dose requirements to induce protection may be prohibitively expensive.

Other groups have tried recombinant protein expression via bacterial organisms. Ning et al. demonstrated that *Salmonella typhimurium* expressing VP28 conferred protection in crayfish against WSSV infection for up to 25 days following oral administration and that the bacteria could be isolated from the animals up to seven days post treatment.[36] However, several problems with using attenuated or genetically modified bacterial expression systems exist. Current methods still require the introduction of live organisms that may have the ability to revert to virulence and may be pathogenic to humans. Additionally, there is a large amount of consumer anxiety over the use of genetically modified organisms and genetically modified bacteria and DNA. Many strategies to select for genetic modification involve the use of antibiotic resistance markers, a growing concern in food borne illness pathogens. This combination of regulatory, safety, and cost hurdles impedes a viable strategy for introducing recombinant protein or protein subunits expressed via bacterial organisms as a prophylaxis into commercial shrimp farms.

RNAi

Exploiting the RNA interference or RNAi pathway has been developed as a novel strategy to mitigate viral disease that requires no protein or protein expression.[46] Instead of administration of a recombinant subunit protein or DNA vaccines expressing a protein, modulation of RNA transcription has been highly successful in preventing mortality caused by a variety of pathogenic shrimp viruses. The RNAi pathway was first characterized in the model nematode *C. elegans* by Fire et al.[12], who was subsequently awarded a Nobel Prize for this significant discovery. RNAi or RNA interference is a term describing a sub-cellular process that results in messenger RNA degradation and subsequent suppression of gene expression in a gene-specific and highly sequence-dependent manner. It is likely that RNAi evolved naturally as an antiviral mechanism in plants and invertebrate species. To modulate gene expression, RNAi triggers, including small interfering RNAs (siRNA) and double stranded RNAs (dsRNA), can be provided exogenously to shrimp to induce this pathway. RNAi has become an invaluable tool for studying invertebrate physiology and host-pathogen interactions. For example, RNAi has been observed as an antiviral response in mosquitoes that transmit human viral pathogens.[19] This has been proposed as a possible strategy to inhibit viral transmission in transgenic mosquitoes expressing an RNAi trigger in the form of an inverted-repeat of the Dengue virus (DENV) genome.[14] This tool would provide special utility in species such as penaeid shrimp, which lack an *in vitro* model or cell culture system for study.

There is evidence that the requisite RNAi machinery is present and functioning in penaeid shrimp in a similar manner to other model invertebrates.[8,47,51] Exploiting this RNAi machinery using dsRNA has been demonstrated to prevent infection or mortality

caused by several different shrimp viral diseases, in both a sequence dependent and independent manner.[21,31,39,50,57] Based on these studies, RNAi is a promising approach to shrimp disease control.[16,20,39,40,46] Specific RNAi triggers have been demonstrated to prevent diseases caused by WSSV[39,61], Yellow head virus (YHV)[49,50,62], Taura Syndrome Virus (TSV)[41] *Penaeus stylirostris* densovirus (PstDNV) (formerly called infectious hypodermal and hematopoietic necrosis virus (IHHNV))[17], and IMNV[30]. Other research examining the ability of dsRNA to not only prevent, but treat both natural and simulated viral exposures has also been reported.[3,17,50,61]

In addition to a being a useful mechanism to exploit for treatment and prevention of viral disease, RNAi has proven extremely useful in elucidating the functions of shrimp gene function. Previous studies have demonstrated a global effect throughout shrimp tissues following inoculation, however, the mechanism facilitating this spread and distribution remains unknown.[21] In *C. elegans*, it occurs via a transmembrane protein called SID-1, a mediator for dsRNA uptake[11] and necessary requirement for global signaling.[58] Recently, the roles of SID-1, Argonaute-1 (Ago-1), and Argonaute-2 (Ago-2) homologues in uptake and processing of dsRNA within shrimp cells have begun to be studied.[22] Ago-1 and Ago-2 are thought to be a critical catalytic component of the RNA induced silencing complex (RISC), the protein that facilitates degradation of target RNA. These genes have been characterized as core components of the RNAi system in some shrimp species, and are thought to be critical in the RNA binding activity of RISC.[22] Additionally, *L. vannamei* Sid-1 (Lv SID-1) was shown to be significantly upregulated along with members of the Argonaute gene family, specifically Ago-2, when shrimp were administered dsRNA of various sequences.[22] This effect was only observed when

duplexes are greater than 50bp in length were administered, and not with siRNAs (20-30bp), likely indicating a necessary length requirement for efficient RNA transport into the cell or assembly into the RISC complex. Double-stranded RNA specific to Lv Sid-1 reportedly causes significant mortality, indicating that it likely has other necessary functions in addition to RNAi facilitation.[22]

Nanoparticle delivery of RNAi

Specific RNAi signaling induces strong antiviral responses, however no feasible delivery vehicle has been developed to exploit this mechanism at a population scale such as in a pond culture system. One preliminary delivery system that showed some efficacy used nanoparticles made of chitosan, a natural product made from deacytlation of chitin extracted from shrimp exoskeleton or shells. These nanoparticles were manufactured using chitosan that had been mixed with VP28 dsRNA, a sequence corresponding to coding regions of subunit envelope glycoproteins of WSSV.[43] Nanoparticles were then top-coated onto feed along with an ovalbumin binder and fed to juvenile *P. monodon.* This preparation conferred 37% increased survival over controls following administration and subsequent challenge with WSSV. This same study also described protection conferred administering feed coated with inactivated DE3 *E. coli* that had been induced to express VP28 dsRNA. Animals administered this feed demonstrated 67% survival over controls following challenge with WSSV, the best protection observed in the experiment.

Chitosan nanoparticles have also shown preliminary efficacy in other arthropod species such as mosquitoes.[65] In a recent study, the authors demonstrate that chitosan nanoparticles can be used to silence endogenous genes in mosquito larvae, including

chitin synthase genes in *Anopheles gambiae* (the African malaria mosquito). Results from this work provide a proof of concept for an oral delivery route for RNAi that could be used to silence both viral and endogenous genes in multiple arthropod species. This paves the way for an entire new generation of RNAi based antiviral therapies for commercially important arthropods such as shrimp. In addition, due to the versatility of RNAi, it could provide the foundation for novel and highly targetable pesticides targeting agricultural pests or disease vectors. To further these goals, additional research into dosage, production of commercial scale quantities of dsRNA, and refinement of administration and delivery protocols needs to be conducted.

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CHAPTER 2: A METHOD FOR *IN VIVO* PROPAGATION FOR THE NECROTIZING HEPATOPANCREATITIS BACTERIUM (NHPB) IN *LITOPENAEUS VANNAMEI*

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Abstract

The necrotizing hepatopancreatitis bacterium (NHPB) is difficult to preserve and has not been propagated *in vitro.* NHPB can be maintained continuously by simply adding specific pathogen free (SPF) shrimp to tanks containing NHP infected shrimp. However, obtaining large amounts of highly concentrated infectious material free of contaminating bacterial flora for repeating challenge experiments was found to be exceedingly difficult using the current published methods. Therefore, a system was implemented using visible implant elastomer (VIE) tags to identify animals within a propagation tank by introduction group. Utilizing this method allowed for continuous reproduction of NHP within propagation tanks and provided researchers with access to infectious material allowing for consistent replication of challenge experiments and concentrated material for preservation.

Introduction

The necrotizing hepatopancreatitis bacterium (NHPB) is a gram-negative, pleomorphic, obligate intracellular bacterium.[3] Phylogenetic analysis of 16S rRNA has placed it in the α-subclass of Proteobacteria, along with such pathogens as *Rickettsia* (~83.5% similarity).[5] Clinical signs of infection include a reduction in feed intake and empty guts, softened shells, flaccid muscle tissue, epicommensal fouling, darkened gills and pleopods, and atrophy of the hepatopancreas organ.[4] NHPB causes a disease in shrimp characterized by a 2-6 week incubation period followed by high mortality that peaks at 34 days post exposure.[8]

In vitro propagation has not been successful, however, NHPB can be maintained continuously by adding SPF shrimp to tanks containing NHP affected shrimp.[7] However, consistently reproducing viable infections from propagation tanks maintained as per Vincent was found to be exceedingly difficult and highly variable. Our observations replicating this method corresponded with previously published data, where only 25% of challenged individuals became infected. Therefore, the purpose of these studies was to establish a target period post infection where individuals with high amounts of infectious material could be identified and used for consistent replication of challenge experiments or preservation.

Materials and Methods

Post larval specific pathogen free (SPF) pacific white shrimp were obtained from the Oceanic Institute, Kailua-Kona, Hawaii. Animals were maintained at 27 ± 2ºC, salinity 30 ± 2 parts per thousand (ppt) in synthetic seawater with constant airstone aeration. Each tank was equipped with a carbon filter and an oystershell airlift biofilter. Shrimp were fed a maintenance diet twice daily (Shrimp Production Formula 45/10, Rangen Inc., Buhl, Idaho). Water quality was monitored weekly by measuring ammonia and nitrite levels (Nitriver 3, Hach Company, Loveland, CO).

Origination and maintenance of NHPB infected shrimp

NHPB infected shrimp were obtained from Amanda Vincent, Gulf Coast Research Laboratory, University of Southern Mississippi, Ocean Springs, MS. Infected shrimp were maintained in 160 gallon tanks containing approximately 80 gallons of artificial salt water, temperature 30 ± 2ºC, salinity 30 ± 2 ppt. Each tank was equipped with a carbon filter, oystershell biofilter, supplemental tank heater, and constant aeration. As animals in the infected tanks died, carcasses and moribund animals were left in the tanks and SPF shrimp were added to replace them as reported by Vincent et al [8] to facilitate transmission by *per os* exposure to infectious animal remains.

In vivo propagation of NHPB infected shrimp

Initially, 3 propagation tanks (E, F, and G) containing 25 animals were challenged per os with infected hepatopancreas tissue. Each time a new introduction group of SPF animals were added to a tank, each shrimp was injected with a visible implant elastomer (VIE) tag (Northwest Marine Technology, Shaw Island, Washington) in the dorsal skeletal muscle of the last abdominal segment. The day each group was added to the tank

was recorded along with the corresponding tag color. The number of shrimp in each tank was maintained at approximately 25 (+/- 5) shrimp. A total inventory of each tank was conducted daily and the color of shrimp which were dead, moribund, or missing was recorded. When dead or moribund shrimp were found with an intact HP, approximately 0.1 g of tissue was collected in a 1.7 mL microcentrifuge tube and stored at -20 ºC for testing by PCR analysis. These data were used as a basis for selection of shrimp likely infected with high levels of NHPB for challenge experiments. Kaplan-Meier survival curves were generated with survival time calculated from the date of introduction until date of death by NHP (FIGURE 1). After 120 days of the experiment a negative control tank was initiated in a room separate and in isolation from the room containing the tanks with NHPB infected shrimp (FIGURE 2). The parameters for maintenance of the negative control tank were identical to those for the tanks containing NHPB. At this time, the inventory level of each tank was increased to 40 shrimp per tank (TABLE 1).

PCR extraction procedure

Total genomic DNA was extracted from each HP sample according to the instructions found in the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, Indiana). Template DNA was maintained at 4ºC to be run in a PCR within 48 hours and stored at -20ºC for long-term use.

PCR protocol

NHP primer sequences, positive control DNA, and PCR protocol were obtained from Dr. Donald Lightner, University of Arizona, Tucson, AZ. NHP PCR was conducted using puReTaq Ready-To-Go PCR Beads (GE Life Sciences, Piscataway, NJ, USA). Primers sequences used were NHPF2: 5'- CGT TGG AGG TTC GTC CTT CAG T-3'

and NHPR2: 5'- GCC ATG AGG ACC TGA CAT CAT C-3'. PCR reactions were performed in a GeneAmp PCR System 9700 (PE Applied Biosystems, Carlsbad, CA, USA) with the following cycling conditions: Step 1: 95ºC for 2 minutes, 1 cycle; Step 2: 95ºC for 30 seconds, 60ºC for 30 seconds, 72ºC for 30 seconds, 25 cycles; Step 3: 60ºC for 1 min, 72ºC for 2 minutes, 1 cycle; 4ºC infinite hold. PCR products were run on a pre-cast 2% Invitrogen E-gel (Invitrogen, Carlsbad, California) in order to confirm product size via comparison to a pGEM DNA ladder (Promega, Madison, WI). DNA was visualized using Gel Doc imaging software (Bio-Rad Laboratories, Hercules, CA).

Histopathology

In order to confirm the presence of NHPB within the tank system, fecal PCR positive animals were fixed whole in Davidson's fixative for 24 hours and then stored in 70% ethanol before being examined by histopathology. Presence of NHPB within the system was confirmed by immunohistochemistry at the Iowa State Veterinary Diagnostic Lab, Ames, IA utilizing techniques described by.[6]

Results

The propagation tanks were established and continually maintained for over 7 months in which SPF shrimp were naturally exposed to shrimp infected with NHPB. Over 1000 individuals in over 120 introduction groups were tracked through the tanks. The mortality rate of shrimp remaining in the propagation tanks was 100% with a maximum time until death of 134 days (FIGURE 1). Antemortem study samples as well as postmortem and moribund tissues were consistently positive for the presence of NHPB as determined by PCR analysis of hepatopancreas tissue. In addition, clinical signs, gross

and histopathologic lesions consistent with NHP were observed in shrimp examined from the propagation tanks. Negative control SPF shrimp held in an isolation room separate from the NHPB propagation tanks had a mortality rate of 30 % with a range until death of 1 day until termination at 173 days. Shrimp removed periodically from the negative control tank were all negative for NHPB by PCR analysis. No evidence of NHP was observed by clinical signs, gross lesions or histopathologic examination of shrimp from the negative control tank.

NHPB was consistently detected in the hepatopancreas tissue from infected tanks by PCR (TABLE 2). In addition, tissues which were removed from infected shrimp for various experiments not reported here[1] were consistently found to be positive for NHPB by PCR and by histopathologic examination. Hepatopancreas tissues collected from shrimp found dead or removed alive from the negative control tank were consistently found negative for NHPB by PCR (TABLE 2). Shrimp removed from the control tank on day 313 and which had been in the tank for 165 days were PCR negative for NHPB and were found to not contain lesions of NHP by histopathologic examination.

Discussion

The purpose of this study was to establish an *in vivo* propagation system for the production of concentrated infectious material. This was done by tracking individual animals using an elastomer tag, coded to an introduction group, thus allowing the duration of the exposure period to quickly be assessed. It involved a *per os* natural exposure providing a closer model of pond conditions within the laboratory. This

infectious material was used consistently for a wide array of studies including development of preservation techniques [2] and challenge models.

During the study period over 1000 individual shrimp in over 40 introduction groups were followed through the tanks and were used to consistently reproduce infections in other experiments by selecting infected individuals in the acute stages of infection that were positive by PCR analysis.[2] Previous long-term maintenance models developed by Vincent et al. utilized addition of SPF shrimp to maintenance tanks every 21-28 days containing populations of approximately 50 individuals. Due to difficulties in reproducing experimental infections in this method, the described modifications were made. In order to increase the likelihood that tissue removed contained high levels of NHPB, individual shrimp were followed throughout the exposure period. The implementation of this method appears to remedy some of the difficulty described by Vincent in obtaining large amounts of infectious material for replicating challenge experiments, where only a quarter of individuals exposed were diagnosed as positive for NHPB following exposure.[7] In order to effectively select individuals during the acute stages of infection, and thus the highest concentration of bacteria within the challenge tissue, animals were sacrificed immediately prior to the period with the highest mortality rate. This period was found to begin at approximately day 18 after introduction and end at approximately day 25 (FIGURE 1 and 2). Outside of this period reproduction of disease in challenge experiments was found to be quite variable. Vincent and Lotz reported a peak in mortality of 34 days which was similar our results of 27 and 34. [8] The time course observed differed somewhat from studies done by Vincent et al [8] when characterizing time course of disease. A longer duration of the chronic disease state was

observed as many PCR positive individuals never succumbed to the disease until well after 100 days of exposure; however this was not confirmed by histopathology. In conclusion, these methods allow for repeatable reproduction of NHP disease within *L. vannamei* from ongoing propagation tanks and enable researchers to have access to a consistent supply of infectious material for challenge models and experiments. This provides for consistent access to inoculum from specific animals with known exposure periods, thus reducing the number of low titer animals in the challenge pool.

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TABLE 1. Number of individual animals added and removed prior to death

	Prior to Control			With Control			
Tank	E	F	G	E	F	G	Control
Total No. Added	100	141	139	221	203	226	81
No. Removed for experiment	20	30	19	30	33[a]	38	34[a]
No. Plotted in Figure 1 and 2	80	111	120	191	170	188	47

[a] includes shrimp that died due to loss of aeration

TABLE 2. PCR surveillance testing results from NHP propagation tanks following initiation of control tank

Tank	# NHP Positive	# NHP Negative	Total Tested	% NHP Positive
G	66	0	66	100.0
F	73	1	74	98.6
E	61	1	62	98.4
Control	0	7	7	0.0

FIGURE 1. Kaplan–Meier survival curve generated with survival time calculated from the date of introduction into propagation tank until date of death by necrotizing hepatopancreatitis (NHP). Shrimp that were removed or died of causes other than NHP were censored at the time time of loss.

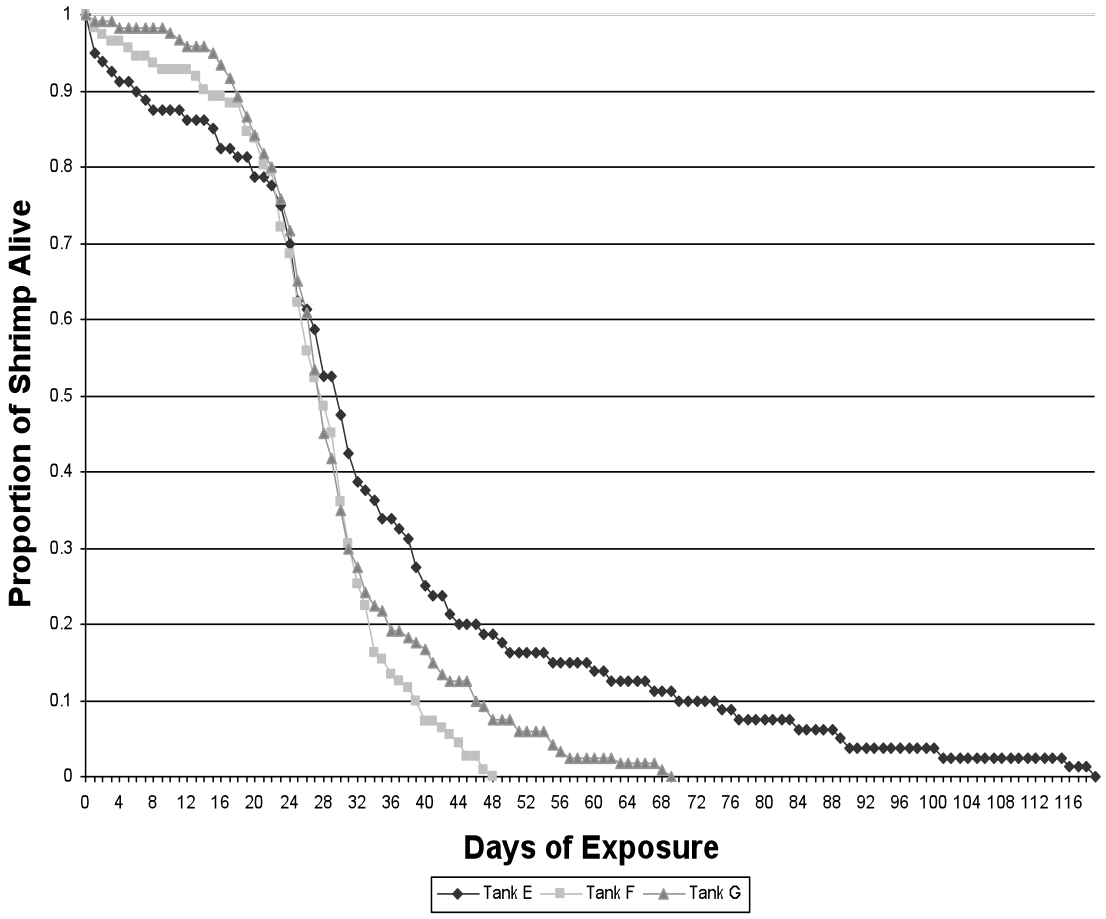
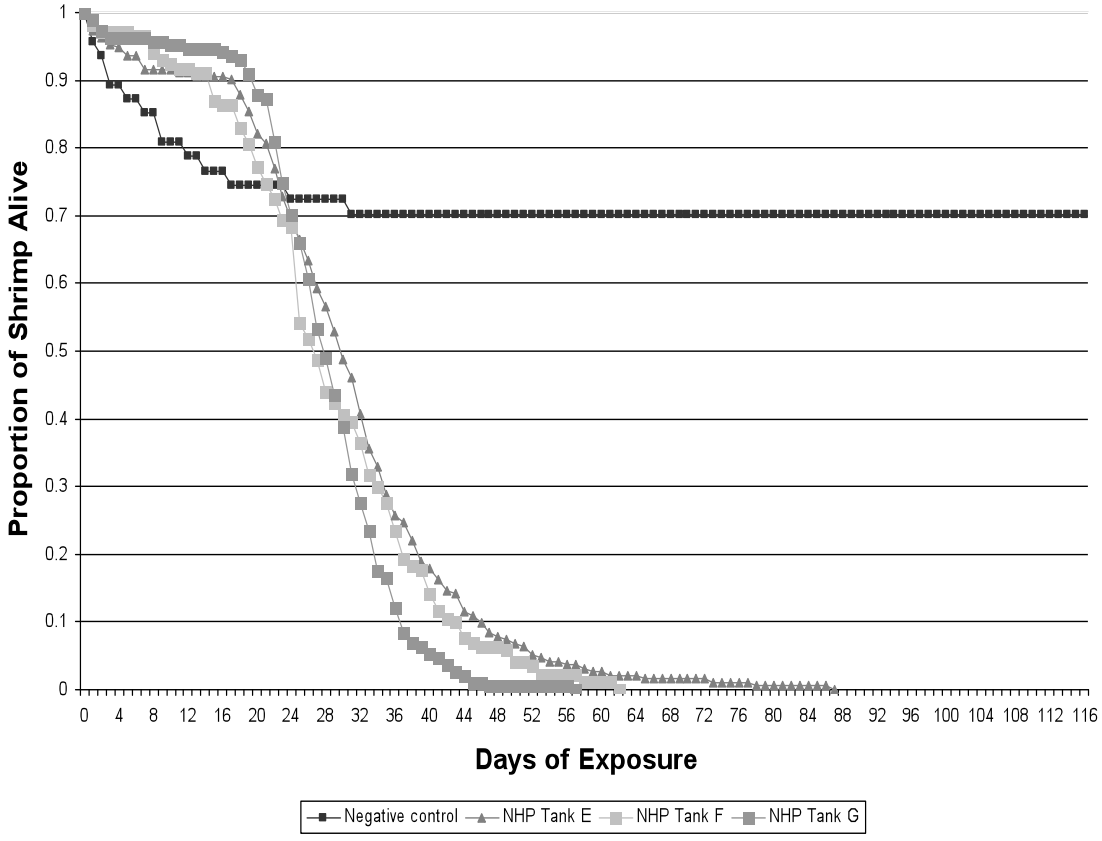


FIGURE 2. Kaplan–Meier survival curve generated with survival time calculated from the date of introduction into propagation tank until date of death by necrotizing hepatopancreatitis (NHP) following addition of the control tank. Shrimp removed for experimental studies or that died of causes other than NHP were censored at the time of loss.



CHAPTER 3. DOUBLE STRANDED RNA PROVIDES SEQUENCE DEPENDENT PROTECTION AGAINST INFECTIOUS MYONECROSIS VIRUS IN *LITOPENAEUS VANNAMEI*

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Abstract

Viral diseases are significant impediments to the growth and sustainability of commercial shrimp aquaculture. New viral diseases continue to emerge and, along with endemic disease, cause significant economic losses to shrimp production. Disease caused by Infectious myonecrosis virus (IMNV) has caused tremendous losses in farmed Pacific white shrimp (*Litopenaeus vannamei*), the predominant species used in shrimp aquaculture, since IMNV emerged in Brazil and subsequently spread to Indonesia. There are no existing antiviral treatments or interventions, outside of pathogen exclusion, to mitigate disease in commercial shrimp operations. Novel RNA interference (RNAi) strategies that employ exogenous double-stranded RNA (dsRNA) are a promising means to approach disease control. Here we describe an iterative process of panning the viral genome of IMNV to discover RNAi trigger sequences that initiate a robust and long-

lasting protective response against viral disease in *L. vannamei*. Using this process, a single, low dose (0.02 μg) of an 81 bp and 153 bp fragment targeting the 5' end of the viral genome was discovered which protected 100% of animals from disease and mortality caused by IMNV. Furthermore, animals that were treated with highly efficacious dsRNA survived an initial infection and were resistant to subsequent infections 50 days later with a hundred-fold greater dose of virus. This protection is highly sequence dependent, because dsRNAs specific to IMNV, but targeting the coding regions for the polymerase and structural protein regions of the genome, conferred lesser or no protection to animals. Interestingly, non-sequence specific or heterologous dsRNA did not provide any degree of protection to animals as had been described for other shrimp viruses. Our data indicate that the targeted region for dsRNA is a crucial factor in maximizing the degree of protection and lowering the dose required to induce a protective effect against IMNV infection in shrimp.

Introduction

Viral diseases that impact cultured shellfish are considerable impediments to successful, sustainable aquaculture practices and trade, and have the most profound economic impact in developing, tropical countries that provide optimal climate and habitat for shrimp aquaculture. The Pacific white shrimp, *Litopenaeus vannamei* (Decapoda: Penaeidae), is uniquely amenable to aquaculture and has rapidly become the dominant farmed shrimp species globally; indeed, production of *L. vannamei* increased from 8,000 metric tons in 1980 to 1.38 million tons in 2004 worldwide.[6] Pathogen translocation and disease outbreaks have ensued with increasing production, and

significant economic losses have resulted with the emergence of each of the major viral pathogens.[29] Epizootics of viral diseases in particular cause substantial mortality in *L. vannamei* and result in devastating financial losses; it has been estimated that annual losses globally as a result of diseases (mostly viral in etiology) have been as high as 3 billion USD.[13,30] The occurrence of a pathogenic virus in a pond of growing shrimp exerts a range of consequences, including forcing the grower to prematurely harvest, increasing production costs through decreased feed conversion, or causing a complete loss of animals.[29] Beyond deliberate pathogen exclusion or selective breeding, which is not effective against all viral pathogens, there are no means to control or treat viral disease and prevent perpetual transmission in farmed shrimp. A practical and efficacious means of protecting shrimp against viral disease would offset these losses and facilitate more profitable production of farmed shrimp in this multibillion dollar per year industry.

RNA interference (RNAi) is promising and emerging approach to shrimp disease control.[9,10,17,18,21] RNAi is a sub-cellular process that results in gene-specific RNA degradation. RNAi triggers, including small interfering RNAs (siRNA) and double stranded RNAs (dsRNA), can be provided exogenously to arthropod cells or whole organisms to study gene function. In recent years, RNAi has become an invaluable tool for studying arthropod physiology and arthropod-pathogen interactions. It is likely that RNAi evolved, in part, as an antiviral response. Indeed, the RNAi phenomenon also has been observed as an antiviral response in mosquitoes that transmit human viral pathogens and has been proposed as a transmission-blocking strategy in which transgenic mosquitoes, expressing an RNAi trigger in the form of an inverted-repeat of the Dengue virus (DENV) genome, no longer support amplification and transmission of the virus.[7]

There is evidence that the requisite RNAi machinery is present and functioning in a similar manner in penaeid shrimp.[4,22,27] Therefore, RNAi is an attractive means to specifically disable virus infection in shrimp cells and thereby protect shrimp from associated pathology and morbidity. Indeed, specific RNAi triggers mitigate disease caused by White Spot Syndrome Virus (WSSV)[17,32], Yellowhead Virus (YHV)[25,26,33] and Taura Syndrome Virus (TSV).[19]

Infectious Myonecrosis Virus (IMNV) is a non-enveloped, double stranded RNA (dsRNA) virus that is a member of the *Totiviridae* family. IMNV was first discovered in 2003 after a severe disease outbreak in 2002 in Brazil that was characterized by high mortality and animals exhibiting necrosis in the tail muscle.[16] In previous outbreaks, mortality has ranged from 40% to 70% prior to harvest.[1] Moribund animals have ever-increasing opaque to white muscle tissue visible through the cuticle, starting in the ventral portion of the muscle and expanding dorsally in each abdominal segment. Histopathologically, animals demonstrate a characteristic coagulative necrosis of skeletal muscle with fluid accumulation between muscle fibers and pronounced hypertrophy of the lymphoid organ due to formation of spheroids, well-delineated non-tubular aggregates of lightly basophilic cells, a feature common to several virus infections that affect shrimp.[16]

IMNV reached Indonesia in 2006[20]; this translocation of the pathogen is evidence for a very real risk for spread throughout Asia and the world. Due to the current and tremendous future potential impact this disease has on the shrimp industry, development of a vaccine or mitigation strategy is prudent. The aim of this work was to discover and optimize RNAi trigger sequences that would elucidate a protective response to IMNV.

We describe an iterative process of panning the IMNV genome for antiviral sequences through which we arrived upon a targeted RNAi trigger for a short, precise portion of the 5' end of the IMNV genome that confers long-lasting and robust protection against IMNV when administered in a single, low dose.

Materials and Methods

Animal Rearing

Specific pathogen free (SPF) postlarvae were received from Shrimp Improvement Systems (Plantation Key, Florida) and reared in a biosecure animal holding facility at Iowa State University. Animals were placed into 1000L Poly tanks containing artificial seawater (Crystal Sea Marine Mix), an oystershell airlift biofilter, and an activated carbon filter. Animals were fed a commercial growout diet until ~5 grams in weight.

Preparation of Virus Inoculum

Based on a previously described methodology[8], tissue macerate from infected animals was diluted 1:3 in sterile TN buffer (0.02 M Tris-HC1, 0.4 M NaC1, pH 7.4) and clarified through three centrifugation steps after which supernatant was removed by subsequent centrifugation: 1) 4000 x g for 30 minutes, 2) 15,000 x g for 15 minutes 3) 25,000 x g for 60 minutes. The final supernatant was diluted 1:10 in 2% saline and passed through a 0.2 micron filter. This stock viral clarification was aliquoted and frozen at -80C and aliquots of this stock were thawed for each viral challenge. Quantitative polymerase chain reaction (qPCR) analysis of RNA extracted following a freeze-thaw cycle indicated 10^5 viral copy numbers/μL were present in the stock.

Determination of Challenge Dose

The optimum dose by injection resulting in infection and disease was tested in 20 SPF animals weighing 8-10 grams. Each animal was injected with 100 μL of stock, 1:10 or 1:100 dilutions thereof, or 2% NaCl (sham) into the third abdominal segment. Animals were observed twice daily for mortality. All virus inoculums resulted in 100% mortality at varying time points. Using these infectious doses, optimum survivorship was observed with the 1:100 dilution, after which 100% of shrimp died by 18 days post infection (dpi) as compared to the stock dilution that resulted in 100% mortality between 1 and 2 dpi, the 1:10 dilution which resulted in 100% mortality at 11 days, or the 1:100 dilution which resulted in 100% mortality at 18 days. The 100-fold dilution of the stock virus was used as the viral challenge dose for the described challenge experiments. Challenged shrimp show characteristic opaque tissue and hypoxic body posturing (FIGURE 1).

Quantitative Reverse-Transcription PCR

Quantitative Reverse-Transcription PCR (qRT-PCR) was performed by using methods adapted from Andrade et al.[1] RNA template was purified using an RNeasy Minikit (Qiagen). Briefly, 30 mg of muscle tissue was dissected from the tail and placed in 600 μL of Buffer RLT. This was then transferred to and mascerated in a ground glass tissue grinder for approximately 100 turns. The tissue mascerate was then homogenized on a QIAShredder column (Qiagen) and purified using RNeasy centrifuge columns according to manufacturer's instructions. 50 μL of RNA template was eluted into RNase free water and stored at -80 °C until the RT-PCR was performed. For the qRT-PCR templates were thawed and boiled at 100 °C for 5 minutes prior to reverse transcription.

One-Step RT-PCR Kit (Qiagen) was used. 2 µL of RNA template was mixed with 5 µL of 5x One Step RT-PCR Master Mix (Qiagen), 1 µL Enzyme Mix, 1 µL DNTP, 0.3 µL IMNV412 (20 uM) (5′-GGA CCT ATC ATA CAT AGC GTT TGC A-3′) , 0.3 µL IMNV545R (20 uM) (5′-AAC CCA TAT CTA TTG TCGCTGGAT-3′), 0.3 µL IMNV p1 (10 uM) (5′-6FAM CCA CCT TTA CTT TCA ATA CTA CAT CAT CCC CGG TAMRA-3′) and 15 uL RNase free water for a total reaction volume of 25 µL. This reaction was then run in duplicate on a BioRad CFX96 Real Time PCR Detection System under the following conditions: RT for 30:00 at 48 °C and 10:00 at 95 °C, followed by PCR for 35 cycles of 15s at 95 °C and 1:00 min at 60 °C. Viral copy number was calculated using CFX Manager Software (BioRad) using an *in vitro* transcribed RNA standard generated using methods similar to Andrade et al[1].

Statistical Analysis

Statistical significance was conducted on mean log viral RNA copies/mL RNA and mean survival at termination of the experiment using One-way ANOVA followed by Tukey's multiple comparison test using JMP 8 Software (SAS Institute Inc. Cary, NC, USA)

dsRNA Preparation

dsRNA was prepared using Ambion Megascript RNAi Kit following the manufacturer's directions. Template DNA for *in vitro* transcription was created by extracting RNA from virus infected animals using a commercial nucleic acid purification kit (Qiagen RNeasy Minikit). cDNAs to IMNV genome were created using specific oligonucleotide primers (TABLE 1) designed from sequences available (GenBank accession no.EF061744)[20] and reverse transcription (Thermoscript RT, Invitrogen). Five

mL of RNA extract was added to the reaction mix and incubated at 50°C for 60 minutes per manufacturer's instructions. Following reverse transcription, template cDNA (~50 ng) was added to a PCR master mix (PuReTaq Ready-To-Go PCR Beads, GE Biosciences) and thermal-cycling was performed using oligonucleotide primers (Table 1) designed to specific regions of the IMNV genome (GenBank accession EF061744.1). Products were then cloned into pCR4.0 vectors (Zero Blunt TOPO PCR cloning kit) and transformed into *E. coli* (TOP10 Invitrogen). Plasmids preparations from these transformants were used as a template source for further PCRs to create DNA clones for RNA transcription. Clones were sequenced and 100% identity with the IMNV genome was confirmed. For synthesis of template for transcription, primers containing a T7 promoter sequence (TABLE 1) on the forward and reverse primer were used to amplify the segments of cDNA with flanking T7 promoter sites. PCR products were subjected to QIAquick (Qiagen) PCR cleanup to remove unincorporated nucleotides, primers, and enzyme. T7 DNA templates were transcribed overnight following the manufacturer's directions. Following transcription, dsRNA products were incubated for 60 minutes with DNase I and RNase, and purified using the provided columns. Transcription of dsRNA was confirmed by gel electrophoresis in comparison with a molecular weight ladder (pGEM ladder, Promega) and the product was quantified by using a spectrophotometer.

Histopathology

Moribund animals that were found prior to death were fixed in whole Davidson's fixative[3] for 24 hours before being transferred to 70% ethanol, In addition, two animals from each tank were anesthetized in an ice slurry and fixed in Davidson's fixative[3],

embedded in paraffin, cut into slides, and stained with hematoxylin and eosin and evaluated for the presence of IMNV lesions.

Shrimp Bioassays

Two hundred liter tanks containing synthetic seawater and an oystershell airlift biofilter were stocked with 10 or 20 SPF juveniles weighing 3-7 grams and allowed to acclimate for 72 hours (h). Tanks were maintained at 28 ppt salinity and 28 °C. Following acclimation, animals were injected intramuscularly at the desired dose and volume of either dsRNA corresponding to regions of the IMNV genome, a heterologous dsRNA control, or a sham vaccination (RNase free water diluent). Following dsRNA administration, animals were challenged with IMNV intramuscularly (~3.2 x 10^3 IMNV copy numbers/μL RNA) into the third abdominal segment at the desired time point (2, 10, or 52 days) or a sham virus challenge (2% NaCl viral diluent) as a strict negative control. Animals were counted daily for mortality. Moribund animals were fixed for histopathology in some experiments. At the termination of experiments animals were euthanized by immersion in an ice water slurry and tissues from experiments were immediately frozen at -80°C. Representative samples at termination were also fixed in Davidson's fixative for histopathology.

Target Specificity

Tanks containing 20 SPF juveniles weighing 5-7 grams were divided into 5 virus challenge groups with three replicates per group. Shrimp were injected with dsRNA constructs corresponding to three different segments of the IMNV genome, a heterologous dsRNA control (eGFP), or a sham vaccination. A total of 2.0 μg of *in vitro*

synthesized dsRNA was inoculated into animals into randomized tanks. Animals were challenged with IMNV 48 hours after dsRNA administration.

Dose Response

Tanks containing 10 animals weighing 3-5 grams were divided into 6 experimental groups that received a dose (2.0, 0.2 or 0.02 μg) of a dsRNA construct (dsRNA 95-475 or dsRNA3764-4805), Three control groups received a heterologous eGFP dsRNA at a single dosage of 2 μg of eGFP dsRNA. Following dsRNA administration, animals were challenged 2 days (data not shown) or 10 days later with IMNV.

Length Dependency

Tanks containing 10 animals weighing 3-5 grams were divided into treatment groups with 3 replicate tanks per treatment. Animals were subjected to injection with one of six dsRNA constructs corresponding to or different truncations of the 95-475 dsRNA (dsRNA95-475, 193-475, 95-376, 194-275, 219-273, or 223-376). A total of 0.02 μg of *in vitro* synthesized dsRNA was inoculated into animals that were challenged 10 days later with IMNV.

Long Term Protection

Shrimp administered 2.0 μg of dsRNA95-475 that survived virus challenge from a prior experiment (n=17) were subjected to a second infection at 100-fold higher dose of virus inoculum (~3.2 x 10^5 IMNV copy numbers/mL RNA), 52 days after the initial virus challenge and 54 days after administration of dsRNA. A naïve challenge control group was injected with the same challenge dosage. Animals were followed for an additional

40 days after secondary challenge. At that time the experiment was terminated and samples taken.

Results

A disease challenge model was established that uses an intramuscular injection of a clarified virus preparation; this protocol yields consistent morbidity, mortality and pathology in infected animals. IMNV challenge resulted in 100% mortality, with variation in duration to mortality according to dose (FIGURE. 1). A 100-fold dilution of the tissue homogenate (~3.2 x 10^3 IMNV copy numbers/µL RNA, data not shown) resulted in 100% mortality over an 18 day period and was used as the virus challenge dose for all subsequent experiments. Quantitative RT-PCR (hereafter referred to as qPCR) provided evidence of virus replication because virus genome copy numbers ranged from 2.5 x 10^5 to 3.5 x 10^7 IMNV copy numbers/µL in muscle tissues sampled at 9 day post-infection when compared with the inoculum dose (3.2 x 10^3 IMNV copy numbers/µL). These qPCR results support histopathological observations, because moribund animals collected from this same experiment showed characteristic IMNV pathology on histopathology sections. Negative control animals that were injected with sterile 2% NaCl (virus diluent) showed no signs of histopathological muscle lesions or trauma at the injection site and the presence of IMNV was not detected in this group by qPCR. The development of this model allowed for a consistent and controlled exposure of virus to facilitate comparison of protection provided by various RNAi triggers.

Three regions that encompass portions of the 5', center and 3' regions of the IMNV genome were initially chosen as targets for dsRNA design (FIGURE 2); these are

designated as dsRNA95-475, 3764-4805, and 5518-6391 according to the range of nucleotides represented from the genome (GenBank accession EF061744). Double stranded RNAs were injected into shrimp in a 2.0 µg dose; animals were infected with IMNV, using the established challenge model, 2 days later. Control shrimp were subjected to heterologous dsRNA or sham-inoculation and subsequently challenged with IMNV, and showed significant mortality with 1.67% and 3.33% survival, respectively after 30 days. Of the animals subjected to IMNV-specific dsRNAs, 81.67% and 61.67% in the dsRNA95-475 and dsRNA3764-4805 groups survived IMNV infection for the duration of the experiment (30 days) (FIGURE 3), respectively; this is a statistically significant difference ($P<0.001$) in survivorship as compared to controls, according to Oneway ANOVA followed by Tukey's multiple comparison test. By contrast, animals injected with dsRNA5518-6391 had mortality similar to the sham-inoculated controls with 5% survival post-challenge. No significant difference was evident between survival in animals injected with dsRNA5518-6391, dsRNA eGFP, or the 2% saline control inoculation at day 30. All of the non-infected controls survived for the duration of the experiment (FIGURE 3). Further, qPCR on muscle tissues sampled from dead individuals at 9-14 days post-infection demonstrated a significant reduction in genome copy number between animals administered dsRNA95-475 or dsRNA3764-4805 (mean 2.44×10^5 and 2.39×10^4 IMNV copy numbers/µL RNA, respectively) when compared with animals administered dsRNA5518-6391, dsRNA eGFP, or sham inoculation (3.44×10^8, 2.1×10^8, and 2.72×10^7 IMNV copy numbers/µL RNA, respectively) (FIGURE 4).

To test the longevity of the protective effect, a group of dsRNA95-475-injected shrimp (n=17) from the initial target specificity assay was subjected to a second infection

at a 100-fold higher dose, 52 days after the initial virus challenge and 54 days after the administration of dsRNA. Animals were followed for an additional 40 days after secondary challenge (94 days from the start of the experiment) and showed 94% survival (16/17), as compared to 10 challenge control animals that showed 100% mortality (10/10) within 8 days.

Double stranded RNAs 95-475 and 3764-4805, which provided the highest demonstrated levels of protection were used in a dose titration study to determine the minimal dose to achieve protection from IMNV challenge. Animals were injected with 2.0. 0.2, or 0.02 μg of dsRNA95-475 or dsRNA3764-4805, then IMNV challenged at 2 d or 10 d post-dsRNA inoculation. As shown in FIGURE 5, 80% of animals receiving dsRNA95-475 were protected from IMNV challenge even at the lowest dose (0.02 μg) and at 10 days post-dsRNA injection. dsRNA3764-4805 elicited this level of protection but only when animals were injected with 10 times the amount of dsRNA (0.2 μg) (FIGURE 5).

Moribund animals were sampled for histopathological analysis from sham-vaccinated controls, eGFP dsRNA administered controls, and the low dose (0.02 μg) of dsRNA3764-4805 at both 2 and 10 d post-dsRNA administration. Evidence of IMNV infection and disease included extensive necrosis of muscle fibers, distributed in small to large irregular foci, not limited to particular muscles but scattered throughout the tail as well as affecting smaller muscle bundles in the cephalothorax and in the legs. The lesions were consistent with histopathological changes previously described as characteristic of IMNV infection.[16] Both acute and chronic lesions were evident, often in adjacent locations. Acute lesions were characterized by coagulative necrosis and fragmentation of

muscle bundles with edema prominent within the muscle bundle sheaths and between affected muscle bundles. Infiltration of hemocytes was mostly limited to small clusters scattered along the connective tissue septa that separated groups of muscles. Chronic lesions were characterized by partial or total loss of myofibers with condensation/contraction of the fibrous connective tissue components. The hypercellular appearance of these bands of fibrous tissue appeared to be due primarily to collapse of the muscle sheaths rather than active fibroplasia. Hemocyte infiltrates in chronic lesions were minimal in contrast to a previous report.[16] Lymphoid organs were evaluated if present in the sections examined. Spheroid formation was evident and usually prominent in all lymphoid organs examined, with multiple spheroids affecting most of the organ. In contrast to histopathological observations in IMNV-infected control animals, muscle lesions in the dsRNA-injected shrimp that were sacrificed at termination of the trial were chronic in appearance. Acute muscle necrosis was not evident in most of these animals, but was limited to a few very small foci in an occasional animal. In addition, two animals that were re-challenged with additional virus and examined by histopathology showed mature fibrous scar tissue but did not demonstrate necrotic lesions.

Because dsRNA95-475 provided significant (P=.0003) in comparison with controls), long-term protection from IMNV-induced disease, and was highly protective even at a low dose, this region was further scrutinized for development of shorter dsRNAs as depicted in FIGURE 2. Two dsRNAs were generated as ~100 bp truncations from the 5' (193-475) or 3' (95-376) ends of the original dsRNA95-475 dsRNA. When administered 10 d prior to challenge, dsRNA95-376 conferred 100% protection, whereas the 5' truncation (193-475) conferred 93.33% protection in animals 30 d post-dsRNA

administration. This survival was statistically significant when compared with the control groups (P<0.0001). Shorter dsRNAs were then designed within this truncated region to the 194-275, 219-273, and 223-376 regions of the IMNV genome (Fig. 2). Of these, dsRNA194-275 and 223-376 injected animals showed 100% survival (P<0.0001) and dsRNA219-273 provided 73.33% protection (P=.0005) following IMNV challenge (FIGURE 6).

Discussion

These studies describe the first use of RNAi to protect shrimp from IMNV, a significant emerging shrimp pathogen; moreover, our data show that very specific, short dsRNAs confer robust and long-term resistance to IMNV-induced disease and mortality. This effect is highly dependent upon sequence selection and the virus gene that is targeted.

Three target regions, spanning the length of the viral genome, were selected as initial targets for dsRNA generation. The first target sequence (dsRNA95-475) corresponds to the N-terminal region of ORF1 frame 1, which is predicted to contain two co-translationally cleaved products, termed Proteins 1 and 2.[22] Protein 1 (93 aa) and 2 (284 aa) are encoded by nucleotides 136-415 and 416-1266 of the IMNV genome within ORF 1 frame 1, respectively, and their functions remain uncharacterized[14]. Protein 1 shares 35% sequence identity with previously described dsRNA binding proteins.[16] In addition, portions of the coding regions for the major capsid protein (MCP) (dsRNA3764-4805) and RNA dependent RNA polymerase (RdRp) (dsRNA5518-6391)[14] also were selected for dsRNA generation. The major capsid protein (909 amino acids) of

IMNV is encoded by nucleotides 2227-4953 within ORF 1, frame 1. The RNA dependent RNA polymerase (736 amino acids) is encoded by nucleotides 5241-7451 within ORF2, frame 3[16]. A non-specific control dsRNA was designed to an exogenous sequence corresponding to enhanced green fluorescent protein (eGFP).[5]

Two of the three dsRNA constructs (dsRNA95-475 and dsRNA3764-4805) conferred significant protection from IMNV-induced disease (FIGURE 3). The dsRNA95-475 that targets the 5'-most end of the IMNV genome provided the greatest protection from disease in terms of dose and duration. dsRNA3764-4805 that targets the sequence that corresponds to the major capsid protein, provides a significant, but intermediate, protective effect (61.67% survival) when dsRNA is administered 48 h prior to virus challenge (FIGURE 3). An intermediate level of protection also was demonstrated with this dsRNA in a dose titration experiment, wherein a ten-fold higher dose was required for protection in comparison to dsRNA95-475 (FIGURE 5). This is an interesting finding because dsRNA corresponding to structural proteins (VP19, VP28) of a large DNA virus (WSSV), are very effective in reducing mortality when administered before challenge.[19]

Specific targeting of the non-structural RdRP protein using dsRNA5518-6391 provided no measurable protection in comparison to the sham-injection used for positive control animals (FIGURE 3). This is in contrast to previous work that demonstrated effective protection from infection when non-structural proteins were the targets of silencing in other RNA viruses such as Yellowhead virus.[25,33]

Dose Response

In order to determine threshold protective doses of dsRNA and simultaneously characterize a dose/response to confirm biological activity of the RNAi response, animals were administered doses ranging from 0.02 - 2.0 μg. A reduction in survival was apparent at the lowest levels for both sequences tested, but even at the lowest doses one sequence (dsRNA95-475), was more protective (80%) (FIGURE 5). This very small dose (20 ng) makes it an ideal candidate antiviral molecule for delivery to shrimp for the purpose of preventing disease caused by IMNV. Previous reports in which dsRNA was used to control disease associated with WSSV infection showed protection with much larger quantities of dsRNA (~ 5 μg/gram of animal or higher) injected per animal[18] and that quantities less than 1 μg of specific WSSV dsRNA were insufficient to protect from mortality.[7] Our studies show that protection is long-lasting for IMNV, even at these lower doses, because dsRNA was effective at disease prevention for at least 10 days as compared to 5 days for YHV when a 25 μg dose was given.[17] This disparity in dose range may be a result of dose-response titration studies that did not extend to the lower limit of what we tested because those authors used 100 μg as a high dose and 7-15 μg as the intermediate dose[19] or extrapolated a dosage based on 5.0 μg of dsRNA being sufficient to suppress endogenous genes[17]. This work demonstrates that vastly smaller amounts of specific dsRNA provide sufficient robust and long-term protection, against IMNV disease (0.02 μg for 10 days). This provides new insight into the capacity of the shrimp RNAi machinery and demonstrates the power of developing RNAi antivirals through target optimization in devising a more cost-effective approach to shrimp disease control.

Target Specificity

The most significant protection against IMNV-induced disease was conferred by dsRNA designed to target a protein-encoding region of the 5'-most end of the IMNV genome, dsRNA95-475. This region encompasses Protein 1 and the N-terminal portion of Protein 2 (FIGURE 2). The precise function of these peptides is unknown, other than that Protein 1 contains a dsRNA binding motif that is postulated to play a role in host immune suppression.[24] Protein 1 may be a crucial gene involved in viral pathogenesis within the host, a factor necessary for early viral replication, or a protein that modifies the host RNAi antiviral response. By analogy, Drosophila C virus and Flock House Virus are pathogenic RNA viruses of other invertebrate species that encode proteins that have dsRNA binding activity and the capacity to modify or inhibit the host RNAi response[12,28]. Protein 1 of IMNV may be fulfilling a similar role in facilitating virus replication by suppressing host RNAi machinery.

Additionally, the accessibility of the targeted portion of the genome to RNAi may be enabling this RNA to more efficaciously inhibit viral genome replication. Significant factors associated with impeding target RNA accessibility include RNA secondary structure and protein occupancy.[15] A study examining small hairpin RNA vectors (shRNAs) targeting conserved portions of the hepatitis B virus genome, found several highly effective targets in a 5' portion of the genome. These 5' targets are much more effective than was predicted because this region contains a highly structured stem-loop, perhaps owing to availability according to genome location.[23]

Length Dependency

These data demonstrate that by optimizing RNAi targets, beginning with larger sequences and moving through gradual truncations, the protective effect of dsRNA, on a per molecular weight basis, increases. However, in the context of the RNAi capacity of a shrimp, a sequence length of greater than 50 base pairs (bp) may be a minimum length requirement, because reduction in survival was observed when the 194-275 (81 bp) dsRNA was truncated to 219-275 (56 bp). This optimum threshold size of the dsRNA corresponds well to a recently published study that suggests that an RNAi trigger in *L. vannamei* has to be greater than 50 bp in length to stimulate transcription of the *Lv*Sid-1 gene, which encodes the membrane-forming protein gene involved in dsRNA transport into cells, and that 50 bp is the lower threshold for a dsRNA to stimulate transcript suppression[11]. In addition, a dsRNA shorter than 50 bp that corresponded to an endogenous shrimp mRNA CDP (shrimp transcript of unknown function) did not stimulate a reduction in transcript in other tissues.[11] Further, dsRNA corresponding to WSSV genes that were shorter than 50 bp did not elucidate protection from virus challenge.[11]

Long Term Protection

The overall data strongly suggest that exposure to the optimized dsRNA produces long-term, specific immunity to subsequent challenge with a single dose as compared to siRNAs that require repeat injections to effect virus clearance.[32] For example, animals subjected to a low dose of dsRNA, and challenged 10 days later demonstrated significant protection against IMNV disease. To further study this phenomenon, animals were re-challenged with IMNV following the initial dsRNA administration and challenge.

Animals that were subjected to a second virus challenge demonstrated high survivorship, even when given much higher doses of virus and after extended periods of time following initial dsRNA administration. This observation suggests that RNAi may serve as a pseudo memory-type response that provides long-term protection from IMNV infection in shrimp. This may also be an indication that once animals are able to initially overcome an IMNV challenge, they are resistant to subsequent challenges. RNAi may be a mechanism that is utilized by the animal to adapt to new viral pathogens and establish resistance to viral replication. This may also help to explain why in field observations, gross lesions of IMN appear to occur concurrently with periods of high environmental stress on animals, such as occurs with high stocking densities or longer grow out periods.[2]

Sequence-Independent Effects

Non-specific dsRNA that was generated to a heterologous target (eGFP) did not have a positive impact on survival in animals challenged with IMNV, in contrast to what has been described with other shrimp viruses including TSV, YHV and WSSV [17,19,31,33]. Heterologous dsRNAs for avian and mammalian immunoglobulin, fish non-coding dsRNA, and bacterial plasmid provided moderate protection against subsequent infection (2 pr 3 days) with TSV or WSSV.[19] Limited protection also was conferred in animals injected with dsRNA corresponding to GFP then infected with YHV. However, this effect was not observed in these studies because no degree of protection from virus challenge was observed in animals pre-injected with dsGFP, even when virus challenge occurred 2 days post dsRNA administration. This may be due to differences in pathogenesis or viral replication mechanisms that IMNV utilizes. Additionally, this

phenomenon may also be explained by differences in genome size; IMNV has a small genome relative to WSSV and TSV, and thus it may have fewer off-target sequences with significant homology to the control dsRNA sequences that are capable of providing this apparent non-specific protection.

Here we describe a process of defining IMNV genome dsRNA targets that provide shrimp protection from virus challenge. Optimization of target specificity, nucleotide length and dsRNA dosage level are key elements that support the potential economical use of RNAi triggers to protect shrimp from viral pathogens. Targets identified in this manner could be utilized in downstream efforts to develop therapeutics or vaccines for shrimp viral pathogens, either newly emerged or pre-existing.

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TABLE 1. Oligonucleotide primer sequences used for dsRNA generation, initial cloning, and qRT-PCR. T7 promoter sequences are underlined.

dsRNA	Sequence 5'-3'
IMNV95T7F	TAATACGACTCACTATAGGGAGAAGAAAGTTTGTTTCGTAGAGC
IMNV474T7R	TAATACGACTCACTATAGGGAGAAAAGGTGGCAGGTGTCCATAC
IMNV3764T7F	TAATACGACTCACTATAGGGAGAAATTTGGGTGGTTGGGACACA
IMNV4805T7R	TAATACGACTCACTATAGGGAGACCCGACTTTCGTGCACAC
IMNV5518T7F	TAATACGACTCACTATAGGGTCAACTCACTCGCAGCTGAAG
IMNV6391T7R	TAATACGACTCACTATAGGGAATATAGCAACGTCGTCTCCG
IMNV193T7F	TAATACGACTCACTATAGGGAAAACCGGAGCTGACCACATTCCA
IMNV194T7F	TAATACGACTCACTATAGGGAAACCGGAGCTGACCACATTCCAA
IMNV275T7R	TAATACGACTCACTATAGGGACTGTGTCACATGTTGCTGCTTCG
IMNV223T7F	TAATACGACTCACTATAGGGACTGTATTGGTTGAGTTCGCAGG
IMNV376T7R	TAATACGACTCACTATAGGGCTGGAGGTGGCAGCATACAAT
IMNV219T7F	TAATACGACTCACTATAGGGCTGGACTGTATTGGTTGAGTTCGC
IMNV273T7R	TAATACGACTCACTATAGGGTGTGTCACATGTTGCTGCTTCGCT
eGFPT7F	TAATACGACTCACTATAGGGAGAATGGTGAGCAAGGGCGAGGAGCTGT
eGFPT7R	TAATACGACTCACTATAGGGAGATTACTTGTACAGCTCGTCCATGCCG
Cloning	
IMNV95F	AGAAAGTTTGTTTCGTAGAGCGAGA
IMNV474R	AAAGGTGGCAGGTGTCCATACTGA
IMNV3764F	AATTTGGGTGGTTGGGACACATGG
IMNV4805 R	CCCGACTTTCGTGCACACAACTTT
IMNV5518F	TCAACTCACTCGCAGCTGAAGGTA
IMNV6391R	AATATAGCAACGTCGTCTCCGCGT
qRT-PCR	
IMNV412F	GGACCTATCATACATAGCGTTTGCA
IMNV545R	AACCCATATCTATTGTCGCTGGAT
IMNVP1	(6FAM) CCACCTTTACTTTCAATACTACATCATCCCCGG (TAMRA)

FIGURE 1. Shrimp survival post challenge with serial dilutions of a stock viral clarification (containing ~3.2 x 10^5 IMNV copy numbers/µL RNA) of infectious myonecrosis virus. Shrimp weighing 8-10 grams were injected with 100 µL of clarification into the third abdominal segment. n=20 shrimp for each viral challenge dose administered.

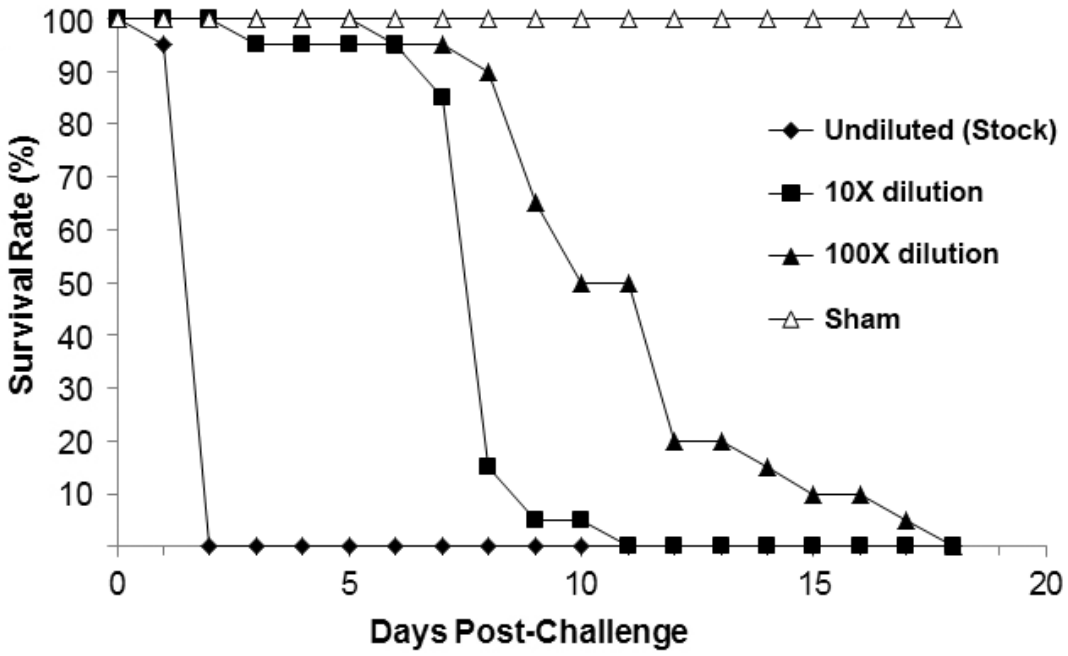


FIGURE 2. Diagram of the IMNV genome transcription and translation products (modified from Nibert (2007) Journal of General Virology) showing regions targeted for RNAi. Predicted protein products are indicated by dark gray lines (Proteins 1-3) or gray shading (major capsid protein and RNA-dependent RNA polymerase). Target regions for dsRNA production are indicated as thick black lines with corresponding nucleotide regions. Longer length dsRNAs that were generated to the region encoding Protein 1 and 2 included two iterations truncated by ~100 bp on either side (194-475 and 95-376). Shorter dsRNAs generated within the Protein 1 encoding region are magnified (bottom left)

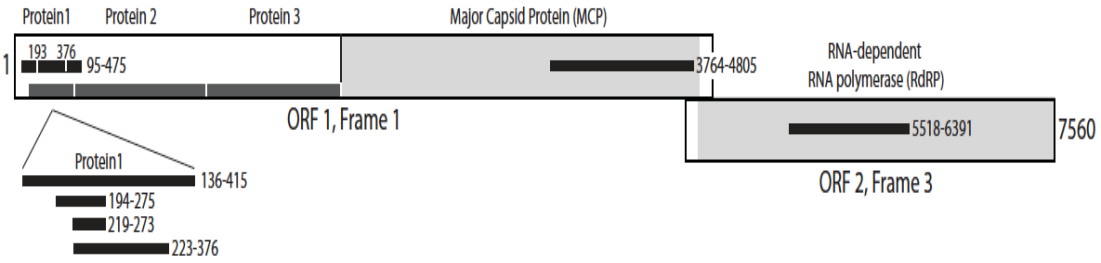


FIGURE 3. Shrimp survival post administration with dsRNA corresponding to different viral gene targets and post IMNV infection. Animals were inoculated with 2.0 µg dsRNA at day 0. Animals were challenged with 100 µL of the 1:100 dilution at day 2. Positive control animals received an inoculation containing an equivalent volume of RNase free water at day 0. Negative control animals received an equivalent volume of 2% saline (viral diluent) on day 2. The dsRNA target position on the IMNV genome or heterologous dsRNA (eGFP) is indicated in the key. X-axis indicates days post dsRNA administration. Y-axis indicates percent survival. 3 replicates of 20 animals (n=60) weighing 5-7 grams were used for each treatment group.

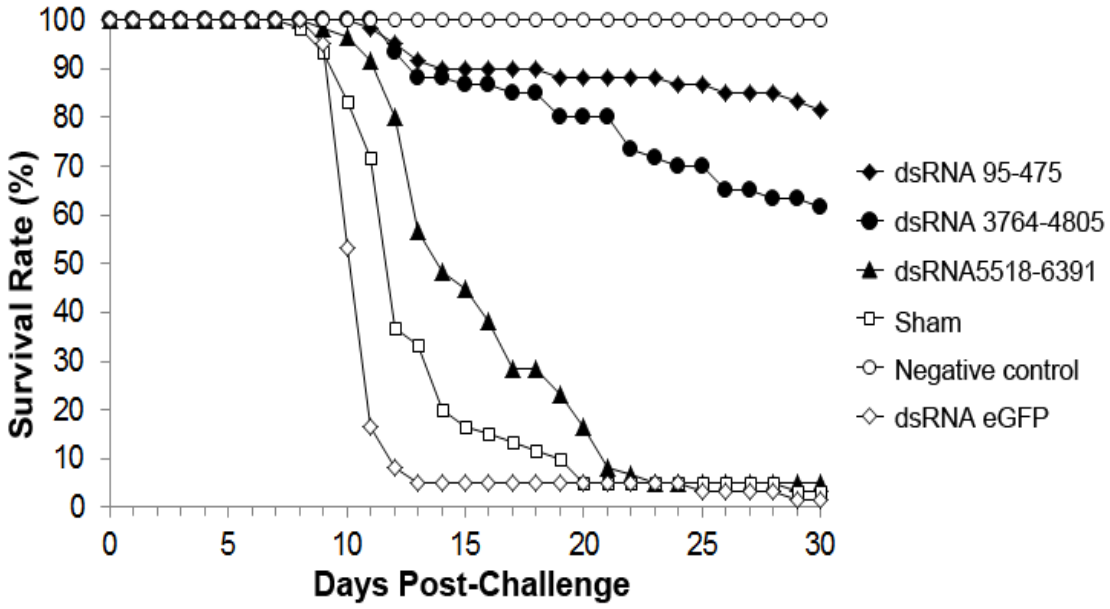


FIGURE 4. Viral copies calculated by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) in muscle tissue at day 9-12 post challenge. X-axis is treatment and Y-axis is log viral genome copy number. Bars represent standard error within the sample.

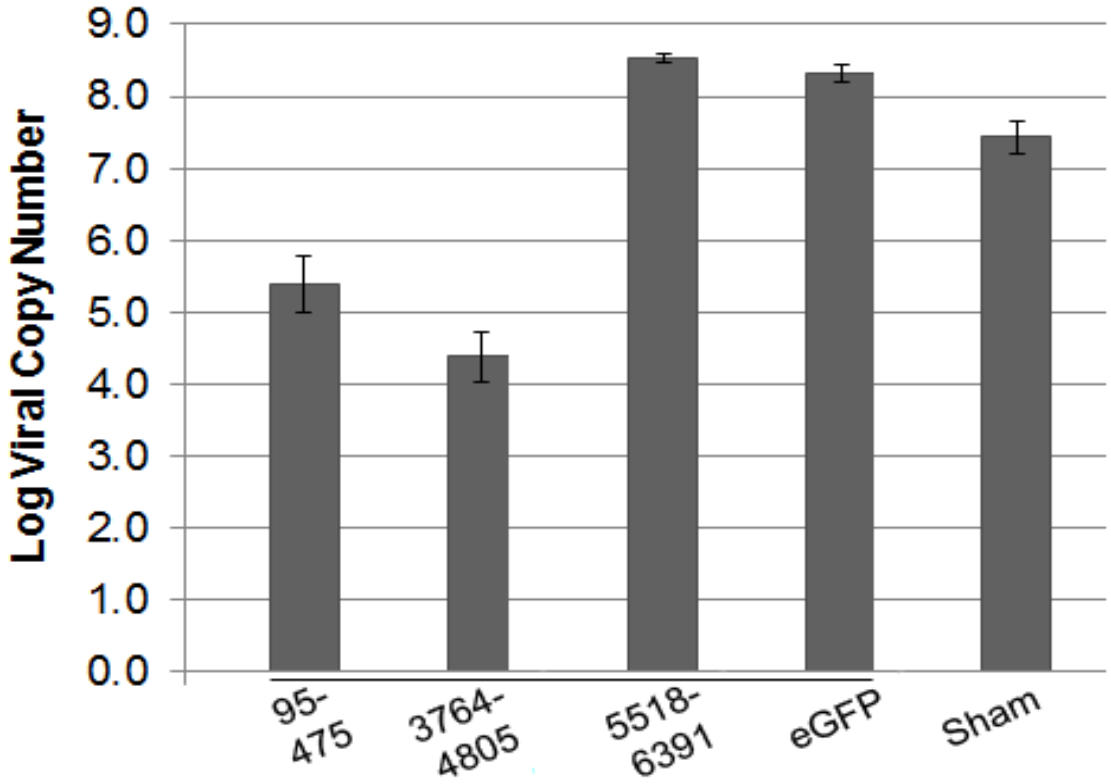


FIGURE 5. A) Shrimp survival post administration with dsRNA 95-475 and post IMNV infection. Animals were inoculated with 2.0 µg, 0.2 µg, or 0.02 µg dsRNA at day 0 and were challenged at day. X-axis indicates days post dsRNA administration. Y-axis indicates survival. 10.B) Shrimp survival post administration with dsRNA 3764-4805 and post IMNV infection. Animals were inoculated with 2.0 µg, 0.2 µg, or 0.02 µg dsRNA at day 0 and were challenged at day 10. C) Shrimp survival post administration with dsRNA eGFP and post IMNV infection. Animals were inoculated with 2.0 µg dsRNA at day 0 and were challenged at day 10. D) Survival of all treatment groups at termination of study (Day 40). Positive control animals received an inoculation containing an equivalent volume of RNase free water at day 0. Negative control animals received an equivalent volume of 2% saline (viral diluent) on day 10. X-axis indicates treatment. Y-axis indicates number of survivors at termination. 10 animals with weights of 3-5 grams were used for each treatment (n=10)

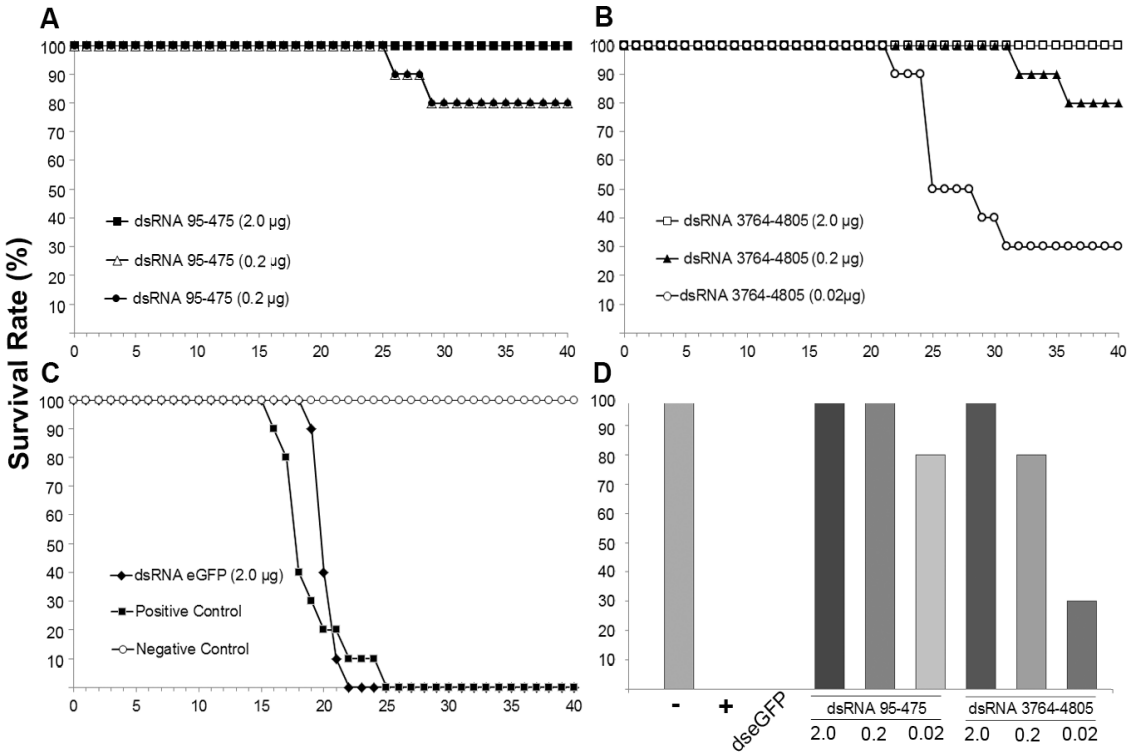
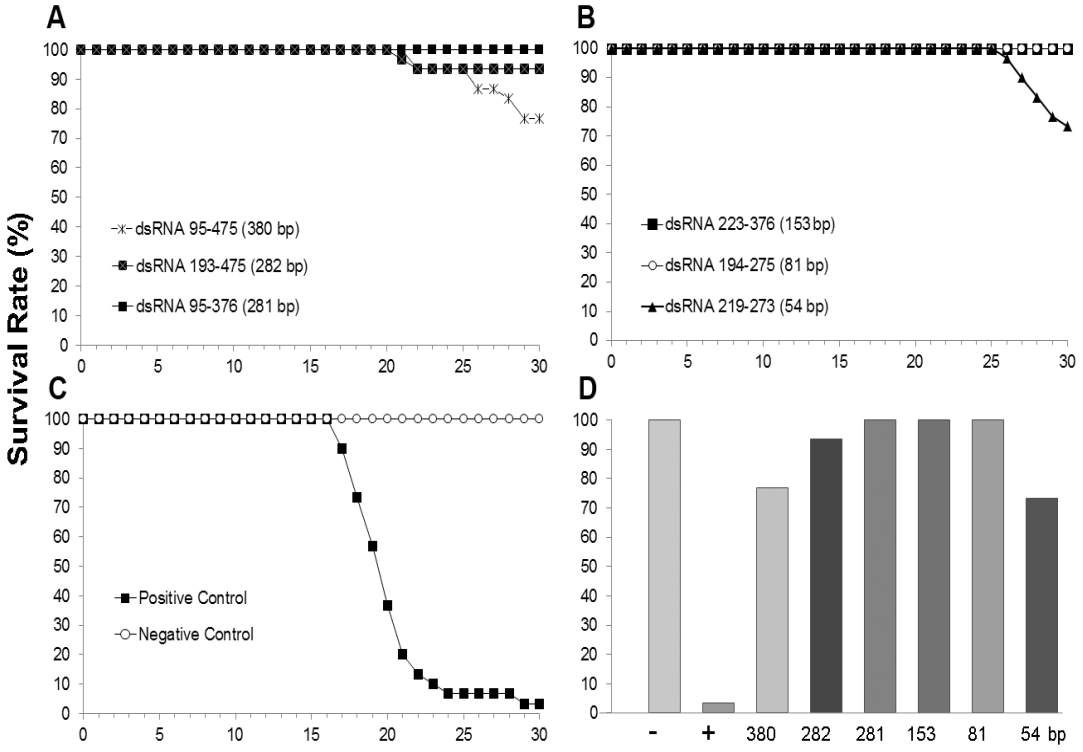


FIGURE 6. A) Shrimp survival post administration with initial dsRNA truncations of 95-475, 193-475, and 95-376 and post IMNV infection. Animals were inoculated with 0.02 µg dsRNA at day 0 and were challenged at day 10. X-axis indicates days post dsRNA administration. Y-axis indicates percent survival. B) Shrimp survival post administration with further truncations of dsRNA 223-376, 194-275, or 219-273 and post IMNV infection. Animals were inoculated 0.02 µg dsRNA at day 0 and were challenged at day 10. C) Shrimp survival of positive or negative controls and post IMNV infection. Positive control animals received an inoculation containing an equivalent volume of RNase free water at day 0 and viral challenge on day 10. Negative control animals received an equivalent volume of 2% saline (viral diluent) on day 10. D) Survival of all treatment groups at termination of study (Day 30). X-axis indicates treatment by dsRNA fragment length. Y-axis indicates number of survivors at termination. 3 replicate tanks of 10 animals weighing 3-5 grams were used for each treatment (n=30) except the negative control which had a single tank (n=10).



CHAPTER 4: SEQUENCE OPTIMIZED AND TARGETED DOUBLE STRANDED RNA AS A THERAPEUTIC ANTIVIRAL TREATMENT AGAINST INFECTIOUS MYONECROSIS VIRUS IN *LITOPENAEUS VANNAMEI*

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Abstract

Infectious myonecrosis virus (IMNV) is a significant and emerging pathogen that has a tremendous impact on the culture of the farmed Pacific white shrimp (*Litopenaeus vannamei*). IMNV first emerged in Brazil in 2002 and subsequently spread to Indonesia causing large economic losses in both countries. No existing therapeutic treatments or effective interventions currently exist for IMNV. RNA interference (RNAi) has been one effective strategy developed to prevent viral disease in shrimp. Here we describe the efficacy of a dsRNA applied as an antiviral therapeutic following viral challenge. The antiviral therapeutic used was a sequence-optimized dsRNA construct targeting a viral sequence encoding dsRNA binding motifs, that had previously showed outstanding antiviral protection when administered prior to infection. Our data indicates that at least 50% survival can be attained with low doses (0.5 μg) of dsRNA if administered 48 hours

following an infection with an otherwise lethal dose of IMNV, and that this degree of protection was not observed when dsRNA was administered 72 hours post infection. Additionally, the administration of this antiviral demonstrated a significant reduction in viral loads in the muscle of animals that died from disease or survived until termination of the study as assessed by quantitative RT-PCR. This data indicates that a sequence and length optimized RNAi antiviral molecule holds promise for use as an antiviral therapeutic against IMNV.

Introduction

Infectious Myonecrosis (IMN) is a severe and emerging shrimp disease that was first described in 2003 after a severe outbreak in northeastern Brazil characterized by high mortality and animals exhibiting necrosis in the tail muscle.[12] The etiologic agent is a non-enveloped, double-stranded RNA (dsRNA) virus that is a member of the Totiviridae family, subsequently named Infectious Myonecrosis Virus (IMNV). IMNV was discovered in Indonesia in 2006;[16] and is causing tremendous impact on the shrimp industry within the country. During outbreaks of IMNV, mortality has ranged from 40% to 70% prior to harvest and causes a dramatic increase in feed conversion.[1] IMNV outbreaks have been found to be associated with longer growout periods and higher stocking densities following an epidemiological survey of risk factors.[2] The insidious presentation of this disease, resulting in a gradual loss of animals over duration of the growout period, makes IMNV an excellent target for a therapeutic agent, as treatments could be initiated immediately following a diagnosis of IMNV within a pond.

RNA interference (RNAi) is one possible and promising method to mitigate viral disease in shrimp.[7,9,13,14,17] RNAi has been used to prevent shrimp viral diseases caused by White Spot Syndrome Virus (WSSV)[13,22] Yellowhead Virus (YHV),[19,20,23] Taura Syndrome Virus (TSV),[15] *Penaeus stylirostris* densovirus (PstDNV) (formerly called infectious hypodermal and hematopoietic necrosis virus (IHHNV)),[8] and IMNV.[11] In addition to administration prior to viral exposure, the therapeutic effect of RNAi has been tested against many different RNA and DNA viruses of animals and humans, several of which are being evaluated in Phase 1 and 2 human clinical trials as antiviral therapeutics.[5] In shrimp, a therapeutic effect has been described against WSSV[22] and YHV[20] post dsRNA inoculation. Some evidence indicates that dsRNA administration and induction of RNAi pathways may allow for clearance of virus in shrimp naturally-infected with *Penaeus monodon* densovirus (PmDV).[3] Recently described methods allow for a sequence and length optimized construct to be developed against a specific shrimp pathogen.[11] The objective of these experiments was to test the therapeutic efficacy of a sequence-optimized dsRNA construct that showed outstanding protection when administered prior to infection (Loy et al. 2011), in the face of an active IMNV infection.

Materials and Methods

Animal Rearing

Specific pathogen free (SPF) *L. vannamei* postlarvae were received from Shrimp Improvement Systems (Plantation Key, Florida) and reared in a biosecure animal holding facility at Iowa State University. Animals were placed into 1000 L tanks containing artificial seawater (Crystal Sea Marine Mix) at 25 ppt salinity, an oystershell airlift

biofilter, and an activated carbon filter. Temperature was maintained at 30º C. Animals were fed a commercial growout diet (Rangen 35/10, Buhl, Idaho) until 3-5 grams in weight.

Preparation of Viral Inoculum

Based on a previously described methodology[6], tissue macerate from infected animals (that tested negative for other shrimp viruses by PCR) was diluted 1:3 in TN buffer (0.02 M Tris-HCL, 0.4 M NaCL, pH 7.4) and clarified through three centrifugation steps after each of which supernatant was removed: 1) 4000 x g for 30 min, 2)15,000 x g for 15 min 3) 25,000 x g for 60 min. The final supernatant was diluted 1:10 in sterile 2% NaCl and passed through a 0.2 micron filter. This stock viral clarification was aliquoted and frozen at -80º C. Quantitative RT-PCR analysis of RNA extracted from a freeze-thaw indicated 10^5 viral copy numbers/μl were present in the stock.

Determination of Challenge Dose

The optimum lethal dose by injection resulting in infection and disease was tested in SPF *L. vannamei* weighing 8-10 g and described in detail by Loy et al.[11] Briefly, each animal was injected with 100 μl of a 1:100 dilution of viral clarification into the third abdominal segment, a dose which resulted in 100% mortality at 18 days post infection (dpi).

Quantitative Reverse-Transcription PCR

Quantitative Reverse-Transcription PCR (qRT-PCR) was performed using methods adapted from Andrade et al.[1] Briefly, 30 mg of macerated muscle tissue and homogenized using a QIAShedder column (Qiagen) followed RNA extraction with an RNeasy Minikit (Qiagen). RNA template (50 μl) was eluted from the column into RNase

free water and stored at -80º C until the RT-PCR was performed. qRT-PCR templates were thawed and boiled at 100ºC for 5 min prior to reverse transcription. RNA template (2 µl) was mixed with 5 µl of 5x One Step RT-PCR Master Mix (Qiagen), 1 µl Enzyme Mix, 1 µl DNTP, 0.3 µl IMNV412 (20 uM) (5′-GGA CCT ATC ATA CAT AGC GTT TGC A-3′), 0.3 µl IMNV312R (20 uM) (5′-AAC CCA TAT CTA TTG TCGCTGGAT-3′), 0.3 µl IMNV p1 (10 uM) (5′-6FAM CCA CCT TTA CTT TCA ATA CTA CAT CAT CCC CGG TAMRA-3′) and 15 µl RNase free water for a total reaction volume of 25 µl. This reaction was then run in duplicate or triplicate on a BioRad CFX96 Real Time PCR Detection System under the following conditions: RT for 30 min at 48°C and 10 min at 95°C, followed by PCR for 35 cycles of 15 s at 95°C and 1 min at 60°C. Viral copy number was calculated using CFX Manager Software (BioRad) using an *in vitro* transcribed RNA standard generated using methods from Andrade et al.[1]

dsRNA Preparation

dsRNA was prepared using Ambion Megascript RNAi Kit following the manufacturer's directions. To create template for dsRNA synthesis, RNA was extracted from virally infected animals using a commercial nucleic acid purification kit (Qiagen RNeasy Mini). Oligonucleotide primers for cDNA synthesis were then designed sequence available (GenBank accession no.EF061744)[16] (TABLE 1). Reverse transcription of RNA was performed using Thermoscript RT per manufacturer's instructions (Invitrogen). Following reverse transcription, template cDNA (~50 ng, not calculated) was added to a PCR master mix (PuReTaq Ready-To-Go PCR Beads) and thermal-cycling was performed using oligonucleotide primers (TABLE 1) designed to specific regions of the IMNV genome (GenBank accession EF061744.1). Products were

then cloned into pCR4.0 vectors (Zero Blunt TOPO PCR cloning kit) and transformed into *E. coli* (TOP10 Invitrogen). Plasmids preparations from these transformants were used as a template source for further PCR reactions to create DNA clones for RNA transcription For synthesis of template for transcription, primers containing a T7 promoter sequence (TABLE 1) on the forward and reverse primer were used to amplify the segments of cDNA with flanking T7 promoter sites. T7 DNA templates were then transcribed overnight (16 hours) following the manufacturer's directions. Following transcription, dsRNA products were incubated for 60 minutes with DNase I and RNase, and purified using the provided columns. Transcription of dsRNA was confirmed by gel electrophoresis in comparison with a molecular weight ladder (pGEM ladder, Promega) and product was quantified by spectrophotometer.

Histopathology

Moribund animals that were found prior to death were fixed in whole Davidson's fixative[4] for 24 hours before being transferred to 70% EtOH before embedding in paraffin. Two animals from each tank were anesthetized in an ice slurry and fixed in Davidson's fixative[4] embedded in paraffin, cut into slides and stained with hematoxylin and eosin and evaluated for the presence of IMNV lesions.

Statistical Analysis

End points of mean survival was analyzed using One-way ANOVA followed by Tukey's multiple comparison test using JMP 8 software. Mean log viral copy numbers ran in triplicate were normalized and then compared following using One-way ANOVA followed by Tukey's multiple comparison test using JMP 8 software

Shrimp Bioassays

Tanks containing 200 L synthetic seawater and an oystershell airlift biofilter tanks were stocked with 10 SPF juveniles weighing 3-5 g and allowed to acclimate for 72 h. Tanks were randomized and three replicates were assigned for each treatment. Following acclimation animals were challenged by injection into the third abdominal segment with lethal dose of IMNV. Forty eight hours later a total of 5.0 μg of *in vitro* synthesized dsRNA was inoculated into animals that had been challenged with IMNV. Sham treatment groups received an equivalent volume of RNase free water. Sham challenge (negative) groups received an equivalent volume of 2% NaCl (viral diluent). Animals were counted daily for mortality and assessed for clinical signs. Moribund animals that were found were fixed for histopathology. At termination 2 animals from each tank were fixed and submitted for histopathological examination. Dead animals removed throughout the experimental period were frozen at -80ºC.

Dose/Duration Bioassay

Tanks were challenged with IMNV virus simultaneously with groups receiving treatments at 24, 48, or 72 h. Treatment groups consisted of either 5.0 or 0.5 μg of *in vitro* synthesized dsRNA 194-475, 5.0 μg of eGFP dsRNA, or a sham treatment of an equivalent volume of RNase free water at an equivalent volume and time interval. Sham challenge (strict negative) groups received an equivalent volume of 2% NaCl (viral diluent).

Results

A disease challenge model was established that uses an intramuscular injection of a clarified virus preparation that resulted in 100% mortality within 18 d, using a 100-fold

dilution of the tissue homogenate (~3.2 x 10^3 IMNV copy numbers/μl RNA Specific pathogen free (SPF) *L. vannamei* (3-5 g in weight) were subjected to a lethal intramuscular challenge and 48 h later to a single dose containing 5.0 μg of dsRNA194-275 demonstrated 50% survival. By comparison animals that received the same dose of eGFP dsRNA (dseGFP) or the equivalent volume of RNase free water showed 100% mortality following challenge (FIGURE 1). Significant differences (P=0.007) in survivorship were evident between the experimental and control groups.

Viral Copy Number Quantification

Differences in virus genome copy number were assessed via qRT-PCR on muscle tissues sampled from dead animals at 8-12 d post-infection or at the termination of the study. A significant reduction (P=0.01) in viral copy number was evident between animals administered 5.0 μg dsRNA194-275 that either succumbed to disease or survived until termination (P<.0001) (mean 5.84 x 10^5 and 4.87 x 10^4 IMNV copy numbers/μl RNA, respectively), when compared with animals that received 5.0 μg of eGFP dsRNA or RNAse free water.

Dosage and Temporal Effects

To further characterize the observed therapeutic response, the temporal effects and dose response factors that influence a successful IMNV therapeutic treatment were tested. Animals treated 24 h after viral challenge demonstrated 60% survival after treatment with 5.0 μg dsRNA (high dose) as compared to 50% survival in the animals treated with 0.5 μg dsRNA (low dose) (FIGURE 3). Control animals that received 5.0 μg heterologous dsRNA eGFP (dseGFP) demonstrated 30% survival and the RNase free

water injected animals showed 0% survival. Animals treated 48 hours post-challenge demonstrated 90% survival in the high dose treatment group and 50% survival in the low dose group with 0% survival in groups receiving dseGFP and 20% survival in the RNase free water injected group (FIGURE 4). Experimental and control groups showed 0% survival when dsRNA was provide 72 h post-infection (FIGURE 5).

Gross Lesions

Animals in each treatment group were examined for gross lesions daily. Presence of gross lesions was evident as single or multiple foci of opacity within the tail muscle. All individuals in all challenged groups developed a focal or multi-focal muscle tissue opacity by day 5 post challenge. Several individuals treated with dsRNA194-275 in the 48 h post challenge had opacities resolve by 12 days post challenge (FIGURE 6). Lesions in untreated animals did not resolve, and all of these animals succumbed to death.

Histopathology

Acute histopathologic lesions were characterized by coagulative necrosis and fragmentation of muscle bundles with edema prominent within the muscle bundle sheaths and between affected muscle bundles. Infiltration of hemocytes was mostly limited to small clusters scattered along the connective tissue septa that separated groups of muscles. Chronic lesions were characterized by partial or total loss of myofibers with condensation/contraction of the fibrous connective tissue components. These lesions correspond with characteristic histopathological changes typically seen with IMNV infection.[11,12]

Moribund animals that were found in tanks were sampled from sham-vaccinated controls (7 animals), eGFP dsRNA administered controls (3 animals), and animals treated with dsRNA 194-275 (4 animals). Additionally, 2 animals from each tank were sampled from surviving animals at the termination of the study (9 animals). Histopathological lesions in moribund animals in animals from all groups were characterized predominantly by extensive fibrosis occasionally with concurrent acute coagulative necrosis. Acute necrosis without chronic lesions was evident in only one animal. Animals treated with dsRNA 194-275 that survived until termination of the study had chronic resolving lesions that varied from extensive fibrosis (2 animals) to scattered small foci of fibrosis (3 animals) or no significant lesions present (3 animals). No acute lesions were seen in any of these longer-surviving treated animals.

Discussion

These studies describe the first use of RNAi to treat disease in shrimp infected with IMNV, a significant emerging shrimp pathogen. Previous data demonstrated that a very specific short dsRNA confers robust and long-term resistance to IMNV-induced disease and mortality when administered prior to viral infection.[11] These experiments demonstrate that the same short dsRNA can also therapeutically treat animals with pre-existing viral infections. Additionally, it was noted that non-sequence specific dsRNA administered at high dosage levels (5.0 μg) does not induce a protective response when administered therapeutically at 48 hours post-infection. This is similar to previous findings that showed no positive effect on survival when dseGFP was administered prophylactically.[11] Demonstrating therapeutic efficacy is a significant step towards

developing an antiviral therapeutic for infected animals as part of a disease control program. Such a protocol could help to eliminate or reduce IMNV loads in infected ponds.

In order to determine threshold therapeutic doses of dsRNA and simultaneously characterize the temporal activity of the RNAi response, animals were administered two doses levels of dsRNA (0.5 or 5.0 μg). Significant differences in survival were apparent at both doses, when given either 24 or 48 h post-virus exposure. This is similar to findings using IMNV specific dsRNA delivered prior to infection, where doses as low as 20 ng protected animals from succumbing to IMNV disease[11] and such a protocol may help eliminate or reduce IMNV loads in infected ponds.

Dose and Temporal Response

Low dosage levels appear to provide protection in animals early in the disease process. By contrast, when dsRNA 194-275 was administered 72 h post-challenge, no survival-enhancing effect was observed. This may be due to a significant amount of viral replication taking place between 48 h and 72 h, where the amount of virus replication that has occurred likely exceeds the capacity of an RNAi antiviral effect. Prior work examining the therapeutic effect of dsRNA on survival of shrimp from YHV or PstDNV challenge has been demonstrated only at higher dosages and shorter intervals (3 or 12 h post infection at a 25.0 μg dose dsRNA[20] or 24 hours post infection with a 5.0-12.5 μg dose dsRNA)[8]. No experimental studies have examined the replication dynamics of IMNV in shrimp, but these data suggest that a large amount of virus replication is occurring between 48 and 72 hours post infection, and after which time animals are refractory to treatment with specific dsRNA.

This temporal phenomenon may also be explained by the target sequence of this dsRNA, a cleavage product called "Protein 1" that contains a dsRNA binding motif.[18] This protein may be involved in suppression of host cellular RNAi responses or innate immunity during early viral replication. Viral proteins with dsRNA binding activity have been described and characterized as inhibiting the RNAi machinery such as Drosophila C virus (DCV) and Flock House virus (FHV).[21,10] If this putative dsRNA binding protein, indeed suppresses an element of the RNAi machinery and it were expressed to a high degree in a large number of infected cells, the ability for the host to elicit an appropriate antiviral RNAi response would be impeded. Further studies into the role of this dsRNA binding protein and the role it may play in immune suppression of the host and pathogenesis during IMNV infection are warranted.

The use of a small and optimized dsRNA sequence and the ability of this sequence to be used as an antiviral therapeutic for IMNV in *L. vannamei* was demonstrated. Small amounts (0.5 μg) of IMNV specific dsRNA can prevent mortality in animals with active viral infection. RT-PCR diagnostic methods have been developed that would allow for a rapid turnaround for a diagnosis.[1] If feasible delivery methods for dsRNA in shrimp ponds become available, a therapeutic treatment of small, targeted dsRNA could provide mitigation of viral disease losses in virus-infected animals.

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TABLE 1. Oligonucleotide primer sequences for dsRNA synthesis, cloning and qPCR

Primer	Sequence 5'-3'	Source
IMNV194T7F	TAATACGACTCACTATAGGGAAACCGGAGCTGACCACATTCCAA	Loy et al (2011)
IMNV275T7R	TAATACGACTCACTATAGGGACTGTGTCACATGTTGCTGCTTCG	Loy et al (2011)
eGFPT7F	TAATACGACTCACTATAGGGAGAATGGTGAGCAAGGGCGAGGAGCTGT	Loy et al (2011)
eGFPT7R	TAATACGACTCACTATAGGGAGATTACTTGTACAGCTCGTCCATGCCG	Loy et al (2011)
IMNV95F	AGAAAGTTTGTTTCGTAGAGCGAGA	Loy et al (2011)
IMNV474R	AAAGGTGGCAGGTGTCCATACTGA	Loy et al (2011)
IMNV412F	GGACCTATCATACATAGCGTTTGCA	Andrade et al (2007)
IMNV545R	AACCCATATCTATTGTCGCTGGAT	Andrade et al (2007)
IMNVP1	(6FAM) CCACCTTTACTTTCAATACTACATCATCCCCGG (TAMRA)	Andrade et al (2007)

FIGURE 1. (Left) Percent survivorship of animals that were infected with a lethal dose of IMNV (day 0), then injected with dsRNA194-275 at 48 h post-challenge. Animals were observed for 20 d post-infection. n=30 animals with 3 replicate tanks of 10 animals per treatment.

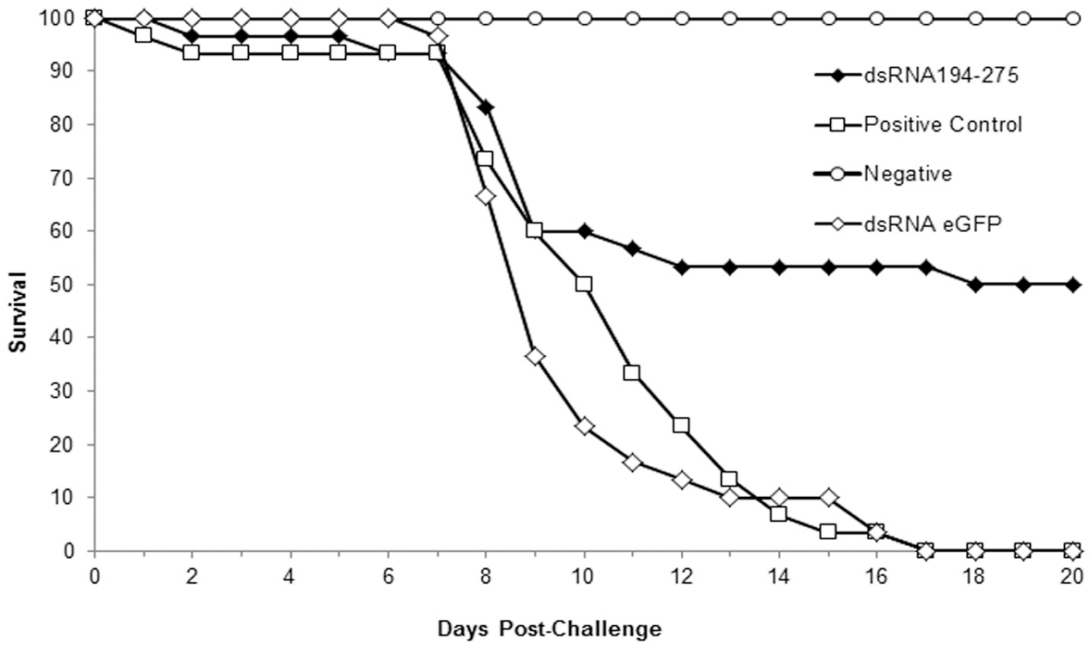


FIGURE 2. Virus copy number, as determined by quantitative RT-PCR, in muscle tissue from IMNV-infected *L. vannamei* that were provided with dsRNA treatment or control conditions. Muscle issues were collected from dsRNA194-275 survivors (Survivors) that survived and were sacrificed at day 20 post challenge. For the dsRNA 194-275 Mortalities, Sham, and dseGFP groups, samples were collected from dead or moribund animals from day 9-14 post challenge. Y-axis indicates mean log viral copy number calculated on The y-axis indicates log viral genome copy number calculated from an RNA standard curve generated per (Andrade *et al* 2007). Bars represent standard error within the sample. n=6 animals per group.

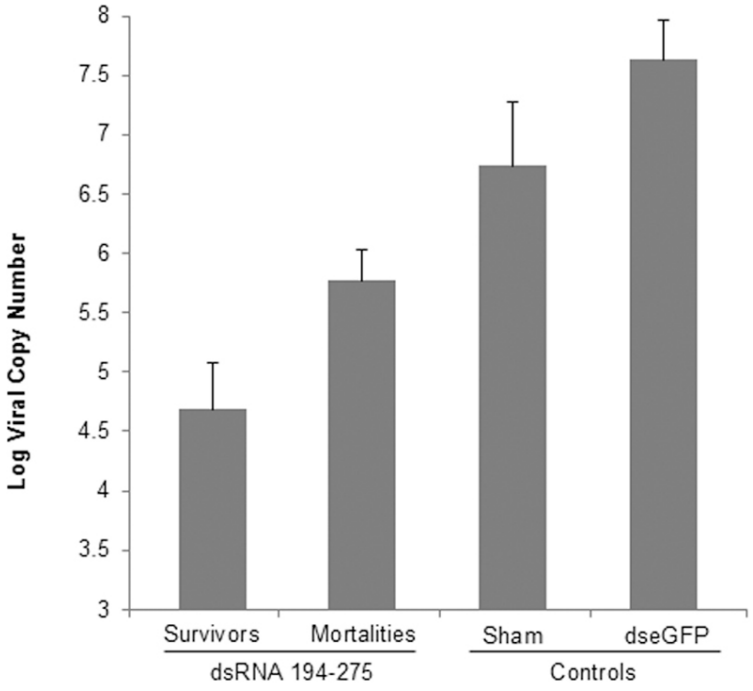


FIGURE 3. Percent survivorship of animals following injection challenge with IMNV inoculum and treatment by injection of dsRNA after 24 hours. X-axis is days post challenge. Y-axis is percent of animals surviving. Sham animals received an equivalent dose of RNase free water and negative control animals were sham challenged with an equivalent dose of sterile 2% NaCl.

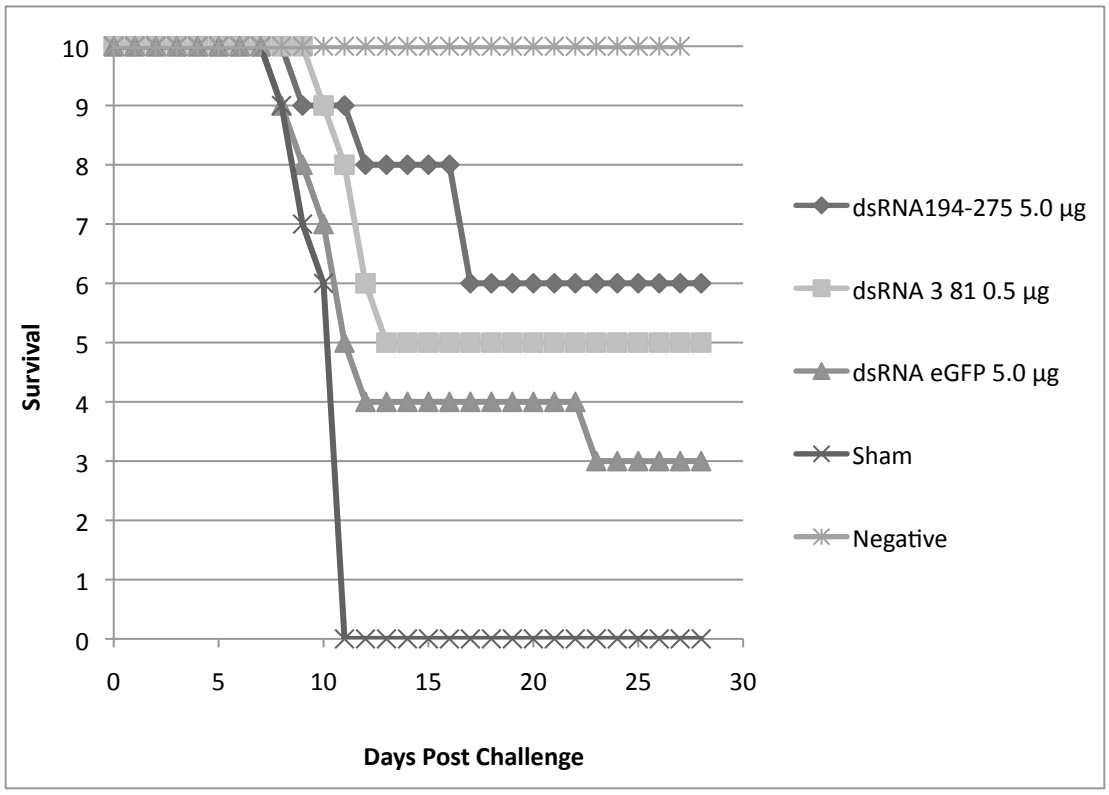


FIGURE 4. Percent survivorship of animals following injection challenge with IMNV inoculum and treatment by injection of dsRNA after 48 hours. X-axis is days post challenge. Y-axis is percent of animals surviving. Sham animals received an equivalent dose of RNase free water and negative control animals were sham challenged with an equivalent dose of sterile 2% NaCl.

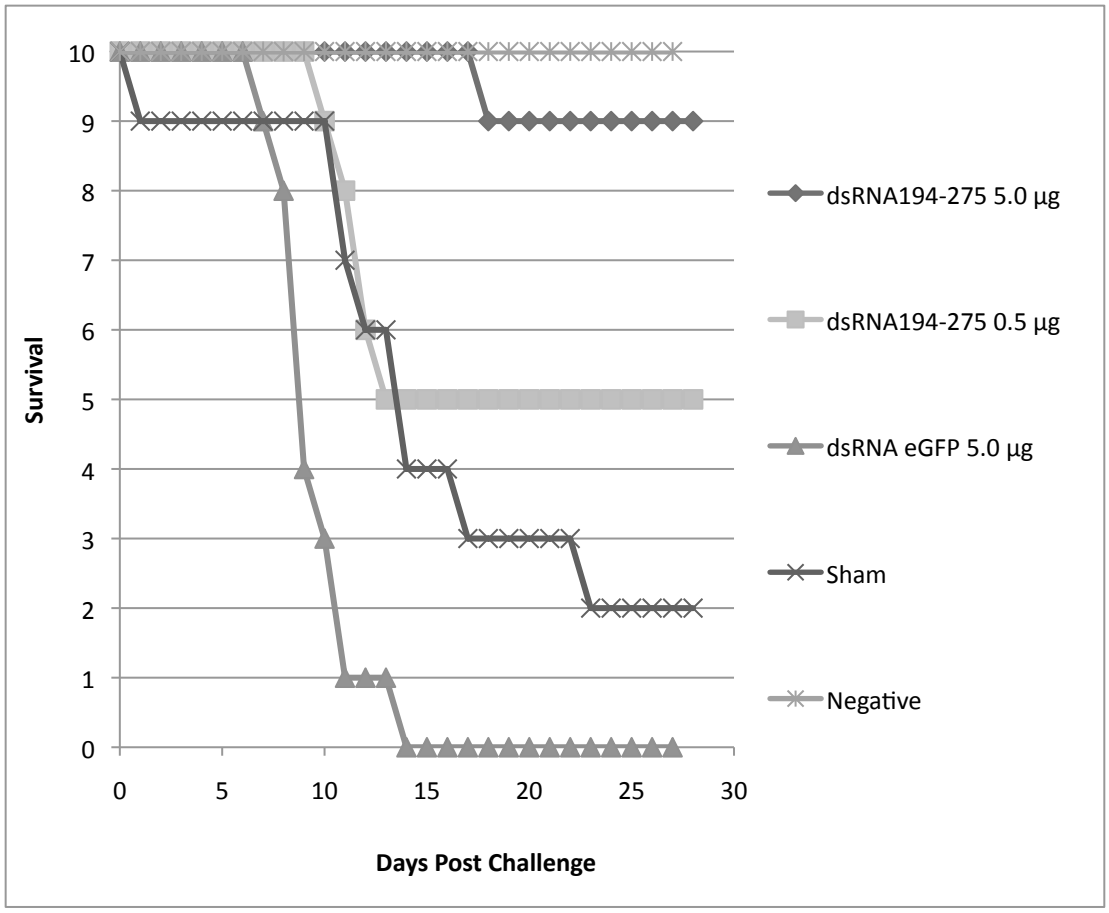


FIGURE 5. Percent survivorship of animals following injection challenge with IMNV inoculum and treatment by injection of dsRNA after 72 hours. X-axis is days post challenge. Y-axis is percent of animals surviving. Sham animals received an equivalent dose of RNase free water and negative control animals were sham challenged with an equivalent dose of sterile 2% NaCl.

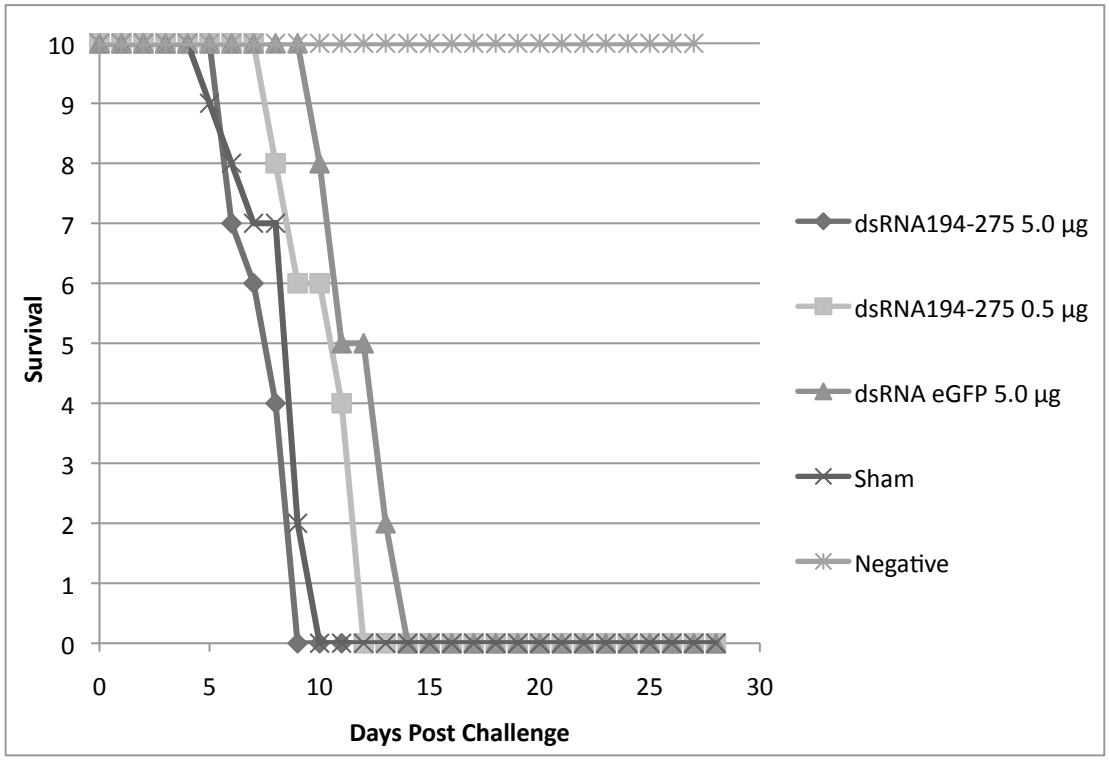
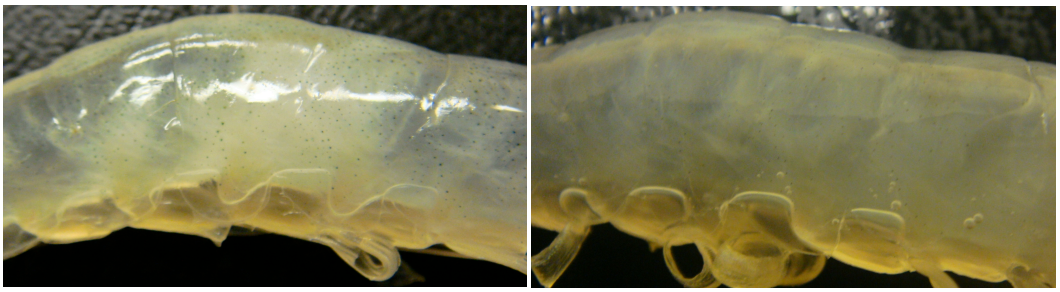


FIGURE 6. Images of gross lesions of the same shrimp abdomen taken on d 4 post challenge (Left) and d 12 post challenge (Right). Focal to multifocal muscle opacities were observed 4 d post challenge and 2 d post dsRNA194-275 administration. Gross lesions were not apparent in animals administered dsRNA at 12 d post challenge and 10 d post dsRNA administration



CHAPTER 5: GENERAL CONCLUSIONS

The results from this work substantiate the idea that the white leg or Pacific white shrimp, *Litopenaeus vannamei*, possess a complex immune system capable of specifically recognizing and responding to insults from viral pathogens. Moreover, that this immune system can be primed or modulated using very small quantities of exogenous double stranded RNA to mount a highly specific, long-lasting, and effective response. Although the mechanisms underlying this response are not well understood, they are likely to be evolutionarily and fundamentally distinct from the specific and adaptive humoral responses observed in vertebrate immune systems. Akin to the adaptive response, the shrimp immune response is functionally capable of eliciting a specific and robust response to viral disease that is functional for extended periods of time, and often that amount to a significant portion of the organism's lifespan. These observations are suggestive of much more complex immune system, one that is distinct from general non-specific innate immunity that is responsive in a generic manner and short-lived. The data presented herein point to RNA interference (RNAi) as a primary antiviral response underlying this long-term immunity. The RNAi antiviral response diverges from the common understanding of proteins as long-term immune response elicitors, the hallmark of "specific" immunity, in that it has instead diverged to recognize nucleic acids, and specific sequence components of those nucleic acids that are produced during a viral infection. In this sense, marine shrimp antiviral immunity is, at many levels, challenging the current paradigms of immunity. This dissertation further expands that body of research.

Specifically, it was observed for the first time in shrimp that very small amounts (20 nanograms) of a precisely targeted dsRNA that elicits an RNAi response can protect animals (100% survival vs. 0% survival) for at least ten days, and likely longer. This is a 100% increase over previously described periods of protection (5 days vs. 10 days) and a 1000-fold lower dose that had been used to demonstrate protection in previous studies.[3] Additionally, shrimp that were subjected to this dsRNA construct then exposed to IMNV, were resistant to subsequent challenges (of 100x higher dosage of virus) for at least 90 days. This same molecule could not only prevent, but also therapeutically treat infections existing infections up to 2 days after receiving a lethal dose of virus. These results indicate that the RNAi antiviral immune response in a shrimp is significantly more potent and longer lasting than had previously been thought.

Several new tools and methodologies were also developed and explored in this dissertation that will enable future work to develop disease control technologies. We developed a concept viral genome panning to develop the most effective antiviral effector molecules is also a novel concept to fight shrimp diseases. Previous work had targeted only a handful of sequences or gene targets against each virus (1 or 2 sequences per virus). This work demonstrates that even within a very small virus such as IMNV (~7500 bp) there are extreme differences observed in survival between the viral sequences targeted (0% vs 100%) and that panning the genome to discover the most protective sequences is a prudent means to develop an antiviral prophylactic. From a production standpoint it was also determined that smaller molecules can also be as effective or more effective than longer ones (80 bp vs. 500 bp) thereby facilitating more efficient production. Additionally, this work also has large implications to developing therapeutic

drugs for shrimp and other invertebrates, because the sequences used to develop antiviral molecules also demonstrated efficacy as a therapy against a pre-exsisting infection.

A lack of *in vitro* tools to explore and mechanistically define the shrimp immune system impedes research into the pathways responsible for the immune phenomena. Not only are marine shrimp a model for a fundamental understanding of invertebrate immunity, but they are of great commercial importance as well. Shrimp culture is rapidly growing, is of tremendous economic importance to many developing countries, and shrimp products are in high demand by consumers. Shrimp production is plagued by viral disease, and limited research and knowledge into the immune response has generated a tremendous deficit in knowledge about the shrimp response to pathogens. Unfortunately, the creation of immortal cell culture lines from marine invertebrates has continued to elude researchers. Therefore, studies on pathogen-shrimp interaction must be done *in vivo* using reliable and repeatable disease challenge models. The work outlined in this dissertation establishes repeatable disease models for a bacterial pathogen as well as a newly emerged viral disease of marine shrimp, to enable such research *in vivo* until such *in vitro* tools are developed. Having a repeatable challenge model and propagation system available for NHP, it is now possible to propagate enough organisms to study these primitive bacteria at a more fundamental level. Currently, only one sequence is available for researchers in GenBank, and it would be prudent to sequence the genome or add to the available sequences of this organism. The analysis of the available sequences suggest that it is a unique and early form of proteobacteria, and sequencing of the NHPB genome may prove not only useful for shrimp farming, but to further a more fundamental understanding of the evolution of early bacterial organisms.

Future Directions

With the models and strategies developed for the study of pathogens in *L. vannamei* established, the way is paved for a fundamental study of genes involved in both viral and bacterial pathogenesis and the host response to these pathogens. Current models of the RNAi machinery and immune pathways in penaeid shrimp are based on an extrapolation from model systems of other invertebrate species including *Caenorhabditis elegans* or *Drosophila melanogaster*. The research presented herein underscores the possibility that a comparatively larger and longer-lived marine arthropod has evolved alternative or enhanced specialized pathways to protect itself in the face of pathogens during the lifetime of the animal, which is significantly longer than most insects and nematodes. Data presented in this dissertation show that the duration of protection against viral agents exceeds that previously described in other animals by weeks or more, and that shrimp have a robust capacity to protect themselves from virus infection for a significant part of its lifespan. It may be that only during severe stress, such as what occurs at high stocking densities and feeding rates, that viruses overcome this immune response and manifest in disease, similar to what occurs to some degree in concentrated vertebrate animal feeding operations. The ability to specifically augment this natural response to specific viral infections would prove a great boon to shrimp farmers throughout the world, who culture their animals in an environment that facilitates and enhances transmission of viruses and leads to devastating disease outbreaks. Research should be undertaken to explore the underlying mechanisms responsible for this duration of specific antiviral protection, and if RNAi machinery is responsible for this "memory."

The discovery process described in this dissertation for a potent and effective anti-viral molecules that can prevent or therapeutically treat shrimp disease caused by IMNV is a critical step in the development of efficacious interventions that can mitigate significant losses producers face from this and other viruses in the field. Lack of knowledge and understanding of the fundamental principles of epidemiology, immunology, and pathology of shrimp viral infection is evident, in that it remains unknown how these signals are initiated, propagated, and maintained during the lifetime of shrimp. A basic understanding of these mechanisms and processes would provide a much-needed background into developing efficient delivery methods. In addition, the varying anatomic and physiologic conditions across the multiple life stages and ages in this species should also be taken into consideration in future mechanistic studies, because fundamental differences in observed responses may occur in this species with that has a broad range of diets and environments.

There is an enormous amount of research needed to help mitigate the impact these viruses have on shrimp production. For example, challenge models that replicate IMN disease dynamics within pond systems and specific triggers that induce acute mortalities or "breaks" in the field need to be established, in the same vein as first chapter of this dissertation as it relates to NHPB disease. This would allow for interventions to be tested in a laboratory under simulated field conditions. Such a model would allow study of the etiology and disease transmission that occurs under pond conditions and would allow researchers to study interventions that may reduce transmission or break disease cycles within culture ponds.

Much of the shrimp culture employs animals derived from a small number of founder stocks. Selectively bred animals do demonstrate antiviral resistance after multiple generations.[1] The genetic basis for viral resistance should be explored because it could reveal new innate immune capacities to tap into for different disease control strategies. Work should continue on evaluating wild stocks for genetic diversity. Genetic resistances to additional viruses using selection pressures on captive animals should be applied to develop animals that are increasingly resistant to viral pathogens and more amenable to intensive rearing conditions. Furthermore, existing disease resistant stocks should be assessed using molecular genetics, to try and establish a genetic basis for immunity and/or resistance to virus induced disease. This knowledge would certainly be applicable to other viruses and diseases in shrimp.

In addition to exploiting the genetic capacity of the shrimp to mitigate disease, interventions must be developed that allow for efficient delivery of molecules to the shrimp. Delivery systems using microparticle or nanoparticle encapsidation of nucleic acids to enhance or optimize delivery across the GI epithelia should be evaluated. Proof of concept work described in the literature review has demonstrated that these RNA antiviral molecules have some degree of efficacy when delivered orally. The next step should be taken to evaluate all available vectors or delivery systems to optimize and facilitate efficient delivery of these RNA molecules through some vehicle. Great strides have been made developing complex and targeted delivery systems for RNA based human drug candidates; such applications of these technologies to shrimp and arthropods would be prudent.

For example, the usage of viral-based delivery systems should be evaluated. Invertebrates are naturally infected with a wide variety of viruses, many of which, such as baculoviruses or alphaviruses are routinely used as laboratory expression vectors. These systems should be evaluated for use in delivering nucleic acid or protein based antivirals to shrimp.

One promising concept utilizes the idea of paratransgenesis, a technique that involves transforming symbionts or colonizing organisms to express a gene that enables disease reduction or disease transmission in the host. One example of this in shrimp used *Artemia*, a common live food source in shrimp hatcheries, as a paratransgenic vector. *Artemia* has shown to bioconcentrate transgenic algae and *E. coli,* and thus may be a vehicle to deliver those transgenic products to shrimp upon ingestion.[3] The usage of a paratransgenic delivery system may be one method of overcoming the difficulties of delivering drugs in marine environments.

In summary, the models for many infectious pathogens *in vivo* have been developed and the molecules needed to induce robust and potent antiviral responses in shrimp have been characterized. Further work needs to focus on methods of delivering these efficiently to the animal in a cost-effective manner. Currently, it is unclear what exact guise or form these delivery systems might take. However, the scientific tools to forge such a delivery system exist and a focused and comprehensive research program will likely be able to overcome this problem in the future.

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"The more we learn about the world, and the deeper our learning, the more conscious, specific, and articulate will be our knowledge of what we do not know, our knowledge of our ignorance. For this, indeed, is the main source of our ignorance — the fact that our knowledge can be only finite, while our ignorance must necessarily be infinite."

Sir Karl Popper
Conjectures and Refutations: The Growth of Scientific Knowledge

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